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14. ABSTRACT
Mild traumatic brain injury (mTBI) and post-traumatic stress disorder (PTSD) are major medical issues for the Warfighter. The current project is designed to evaluate the impact of mild traumatic brain injury (using blast overpressure) and the processes involved in traumatic stress (using a predator exposure procedure and a conditioned fear procedure) in a rodent model. The studies evaluate these insults alone and in combination to specifically address the question of whether mTBI can exacerbate the effects of psychological stress. Additionally, following the insults, a molecular biological evaluation is performed based upon the discovery of biomarkers that have been shown to be correlated with other forms of TBI. Thus, the project aims to systematically assess the combined effects of blast overpressure, traumatic stress and learned stress responses in rodents with the aim of understanding how these forces may interact to impact behavior as well as evaluating their outcome on known biomarkers involved in TBI and stress response system activation.

15. SUBJECT TERMS
Traumatic brain injury, post-traumatic stress disorder, blast over pressure

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INTRODUCTION:

Mild traumatic brain injury (mTBI) and post-traumatic stress disorder (PTSD) are medical issues for the war fighter. Sometimes, mTBI and PTSD present a convergence of symptoms, making it difficult to distinguish between the behavioral manifestations of the two conditions and to determine the extent to which the processes of traumatic stress and mild brain injury might be related. The current project is designed to evaluate the impact of these two insults in rodent models. To model the effects of mTBI, we are using a blast over pressure (BOP) procedure. Two different procedures are used to model traumatic stress / PTSD. First a predator exposure procedure is used to present a traumatic stress event to the rat; second, a conditioned fear procedure is used to model a process known to be disrupted in PTSD. Notably, the studies evaluate these insults alone and in combination to specifically address the question of whether mTBI can exacerbate the effects of psychological stress. The studies are focused on evaluating the short- and long-term behavioral impacts from the insults, and use dependent measures from procedures including operant performance, conditioned suppression (conditioned fear), Morris water-maze and elevated plus maze. Following the insults and the behavioral testing, a molecular biological evaluation is performed based upon the discovery of biomarkers that have been shown to correlate with other forms of TBI. Thus, the project aims to systematically assess the combined effects of blast overpressure, traumatic stress and conditioning responses in rodents. The overall aim of the project is to increase our understanding of how these challenges interact to impact behavior and how they are reflected in known biomarkers involved in TBI and stress response system activation.

KEYWORDS:

mTBI, PTSD, animal behavior, rat, conditioned fear, stress, biomarker.

OVERALL PROJECT SUMMARY:

Task 1: Generation of approved IACUC protocols.

Two protocols were generated and approved by the WRAIR/NMRC IACUC and subsequently approved by the MRMC ACURO for the conduct of the studies in this report.

Task 2: Evaluation of combination BOP and predator exposure on (a) Morris water maze (n=40) and (b) elevated plus maze (n=40) with subsequent biomarker assay.

In task two, we performed two studies to evaluate combination BOP and predator exposure.

Task 2, study 1, Evaluation of combination BOP and predator exposure on the Morris water maze (MWM).

In the first study we assessed the effects of repeated exposure to BOP and stress on cognition (spatial working memory) using a MWM task. Four treatment groups were used as defined in the table below. The treatment conditions were chosen to evaluate mTBI from BOP, and a psychological stressor, from predator exposure, alone and in combination. Additionally, a control group received sham BOP and sham predator exposure treatments.

Table 1 Treatment conditions for Task 2, study 1.

Group	mTBI	Stressor	n
Control-sham	sham	sham	10
Control-Predator	sham	Predator Exposure	10
Blast-sham	BOP	sham	10
Blast-Predator	BOP	Predator Exposure	10

The experimental design is illustrated in Figure 1 below. BOP (or sham) exposures (one per day under anesthesia) were at the 75 kPa intensity where rats are facing the blast wave inside the shock tube (see appendix 2, Methods and Procedures). Approximately 4 h after the BOP exposure, rats were exposed to a predator in a protected fashion or sham (see appendix 2, Methods and Procedures). That is, each day when a BOP was presented, a different predator exposure was presented. The order of predator exposure was snake, ferrets and cats. On the day following the last predator exposure, MWM sessions were conducted. Following MWM evaluation, animals were euthanized and tissue samples were collected for analysis (see appendix 2, Methods and Procedures).

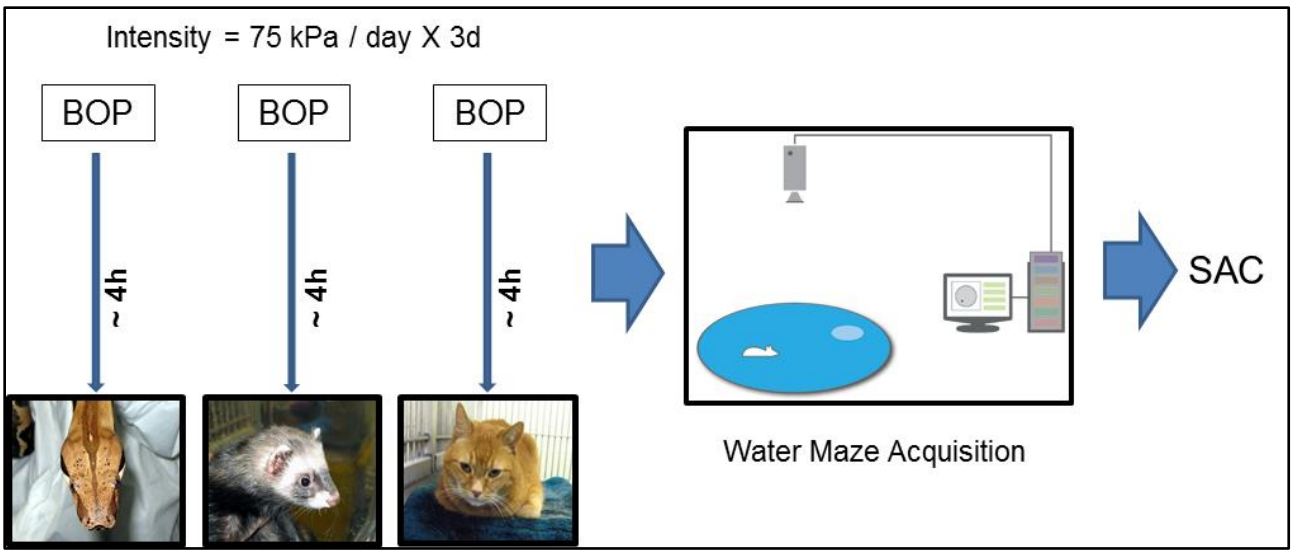

Figure 1. Experimental Design for the study to evaluate BOP effects on Predator exposure evaluated with the Morris Water Maze.

Behavioral Results Task 2, study 1.

Figure 2 shows the main results from the study. All rats learned the MWM. That is, with successive trials, all rats showed a substantial decrease in the latency to find the target. A comparison of the treatments (between groups) did not show a significant effect. Thus, there was no difference in the ability of the rats to learn the maze.

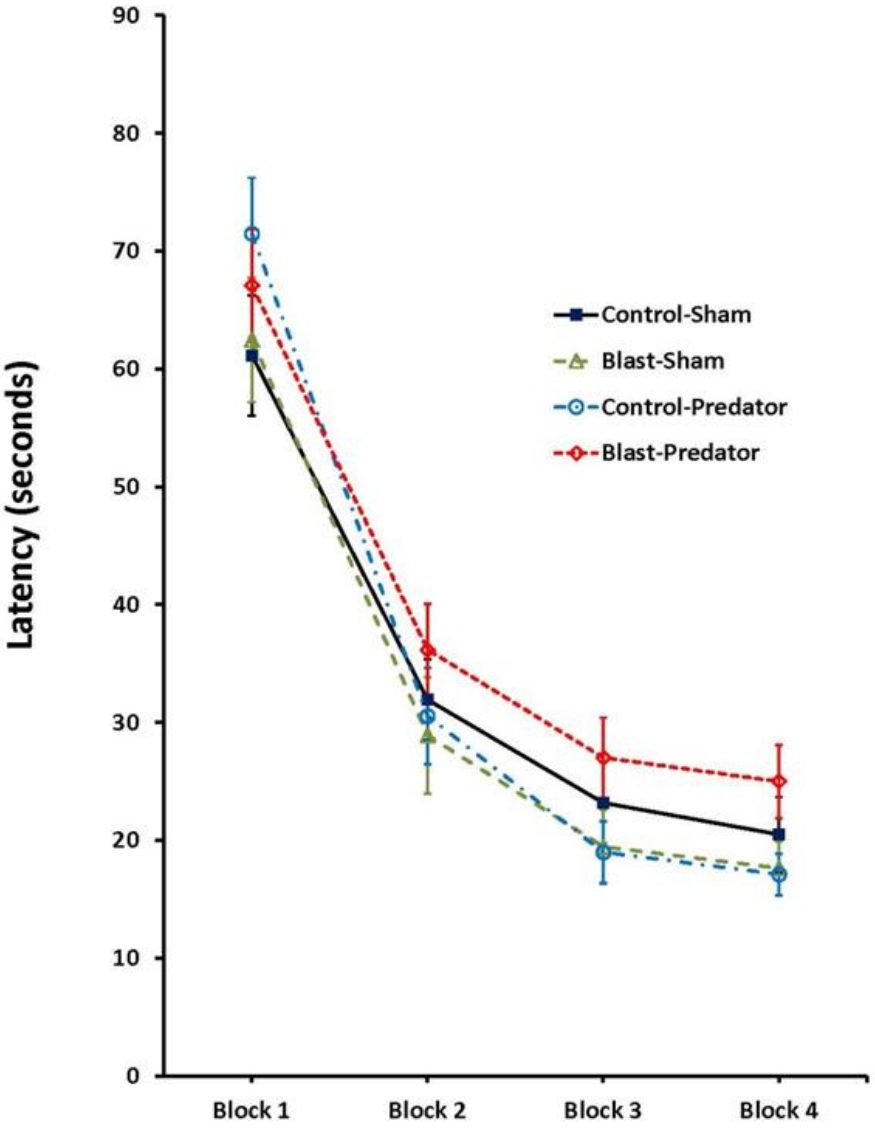

Figure 2. Acquisition of a MWM task in rats after BOP and / or Predator exposure. Ordinate: Average latency to reach the goal platform on the maze. Abscissa: Consecutive testing blocks. Each point represents the means (+/- SEM) from 10 rats.

Biochemical Results Task 2, study 1.

In brain tissues from groups tested for Morris Water Maze (MWM) deficit, α-II spectrin and SBDP-145/150 were readily detectable in this group 48h after the last BOP, but did not indicate any change in abundance after sham predator + sham BOP (SS) or sham predator + BOP (SB) (data not shown). Analysis of plasma derived from sham predator + sham BOP (SS) and sham predator + BOP (SB) groups indicated that concentration of amyloid beta (Aβ) 40 (82-86 pg/L) was greater than that of Aβ 42 (7.5-7.8 pg/L), but

the relative concentration of both peptides were unchanged in serum after SB treatment compared to that of SS treatment (Figure 3).

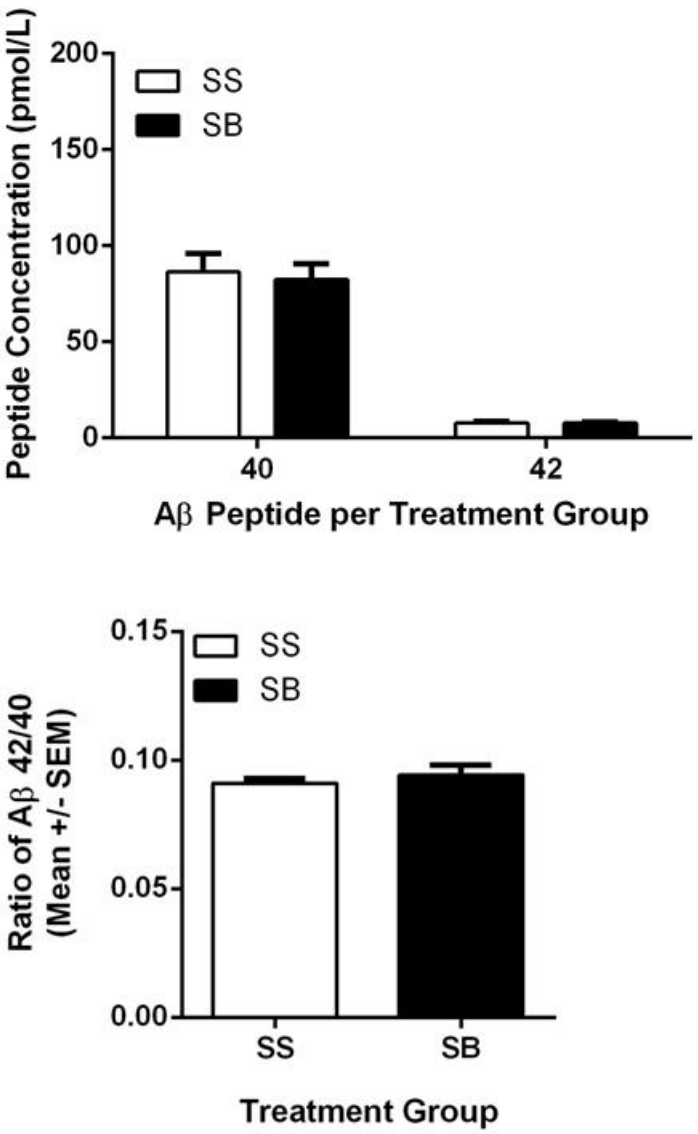

Figure 3. Quantitative ELISA of Aβ 40 and Aβ 42 in plasma. Plasma samples are from rats in Experiment 2 and were sacrificed 48h following the last BOP exposure and MWM analysis. (Top) Quantitation of Abeta (Aβ) peptides 40 and 42. (Bottom) The ratio of Aβ 42 / Aβ 40. Data is shown as the mean +/- SEM (N = 5/group).

Discussion and Conclusion, Task 2, study 1.

All rats in all groups learned the MWM. The intensity of the BOP that we used in this study was, apparently, insufficient to produce the spatial memory disruption that is evaluated with the MWM and is observed with more severe TBI. The predator exposure also did not disrupt performance on the MWM. This result is not surprising as an acute

traumatic stressor does not, typically, produce a disruption in spatial memory task performance. It is notable, however, the combination of BOP and predator stress did not have a synergistic effect to the extent of causing a behavioral deficit since the performance of this treatment group was not statistically different than the other treatment groups, including sham controls.

There was no effect on SBDP-145/150 in brain tissues or amyloid beta peptides in the plasma. The intensity of the treatments is likely too low to induce an acute (< 7 days) effect.

Task 2, study 2, Evaluation of combination BOP and predator exposure on the elevated plus maze (EPM).

In the second study for task 2, we assessed the effects of repeated exposure to BOP and predator stress on activity using an elevated plus maze. Four treatment groups were used as defined in the table below. As in Task 2, study 1, the treatment conditions were chosen to evaluate mTBI from BOP, and a psychological stressor, from predator exposure, alone and in combination. Additionally, a control group received sham BOP and sham predator exposure treatments.

Table 2 Treatment conditions for Task 2, study 2.

Group	Stressor	mTBI	n
Pred + BOP	Predator Exposure	BOP	10
Pred + sham	Predator Exposure	sham	10
sham + BOP	sham	BOP	10
sham + sham	sham	sham	10

The experimental design is illustrated in Figure 4 below. As in study 1, BOP (or sham) exposures (one per day under anesthesia) were at the 75 kPa intensity where rats are facing the blast wave inside the shock tube (see appendix 2, Methods and Procedures). Also, as in study 1, approximately 4 h after the BOP exposure, rats were exposed to a predator or sham in a protected fashion (see appendix 2, Methods and Procedures). That is, each day when a BOP was presented, a different predator exposure was presented. The order of predator exposure was snake, ferrets and cats.

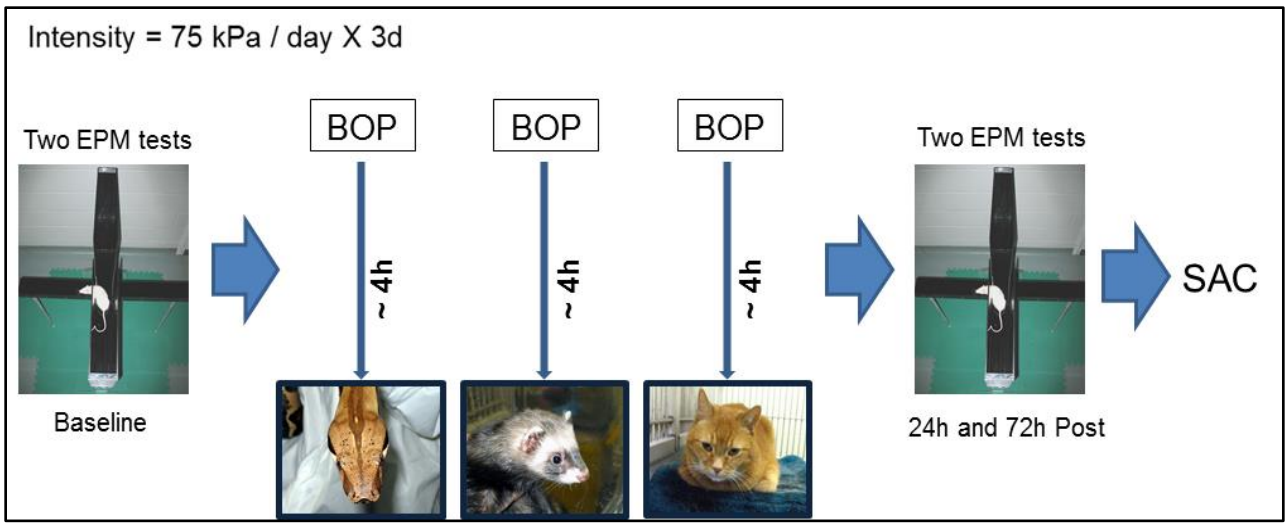

Figure 4. Experimental Design for the study to evaluate BOP effects on Predator exposure evaluated with the Elevated plus maze.

Details of the EPM appear in appendix 2, Methods and Procedures. While several measures were collected for this procedure, the main dependent measure that we used was basic activity (i.e., movement counts). Other measures produced similar effects as basic movements or did not yield systematic effects. Rats were evaluated twice on the EPM during the week before any exposures (BOP or predator) took place and data from these sessions were averaged and treated as baseline. Approximately 24 hours and 72 hours after the last BOP and predator exposures, rats were again evaluated on the EPM. Following the second EPM testing, rats were euthanized and tissue samples were collected for analysis (see appendix 2, Methods and Procedures).

Treatment impact was characterized as a difference in EPM performance between baseline and post-treatment testing (i.e., difference scores). Using these data, a two factor, mixed model, repeated measures, ANOVA was used to evaluate statistical significance. The between groups factor was treatment condition (4 levels as illustrated in Table 2) and the within-groups repeated factor was time (2 levels, 24 h and 72 h tests as illustrated in Figure 4). ANOVA and contrasts were performed using SAS analytical software (Proc Mixed) and a Satterthwaite approximation for the denominator degrees of freedom was employed. Several covariance structures were tested for fit using AIC and BIC measures resulting in the choice of a compound symmetry covariance structure for the model (Kincaid, 2005; Littell et al., 2000; Wolfinger & Chang, 1995). Probability values < .05 were treated as statistically significant.

Behavioral Results Task 2, study 2.

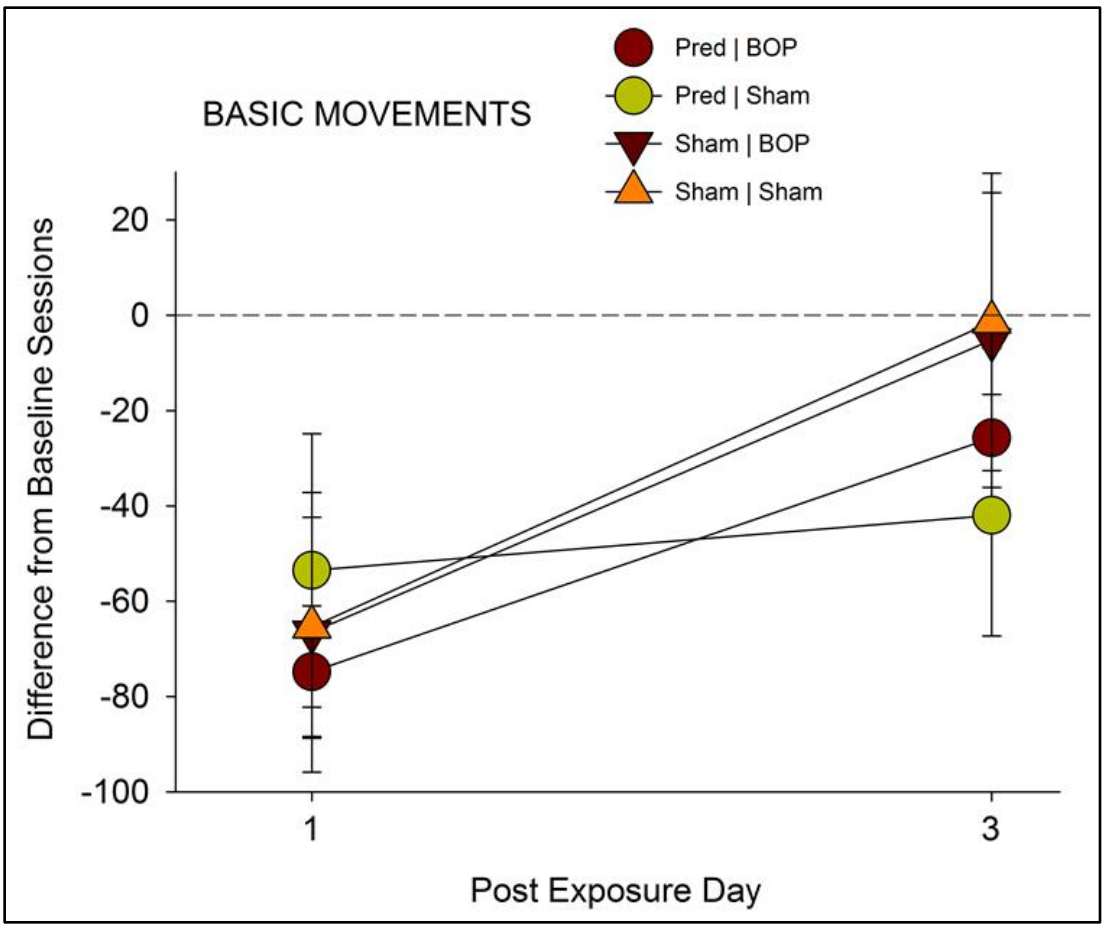

Figure 5. Effects of BOP and / or Predator exposure on the EPM tests. Ordinate: Movement counts as a difference score from baseline. Abscissa: Post treatment time point. Each point represents the means (+/- SEM) from 10 rats.

Figure 5 shows the main results from the study. A decrease in exploratory behavior was observed in all treatment groups when tested 24 h after exposures. ANOVA revealed a significant effect for time ($F[1,36]=18.46$, $p<.0001$). Neither the group effect nor the time x group interaction, however, was statistically significant. Recovery to baseline was observed in some groups during the 72 h test. In this regard, the two treatment groups that did not receive the predator exposure (sham + BOP and sham + sham), on average, showed the greatest amount of recovery and, at the 72 h test had, essentially, returned to baseline activity performance. Tests of the effect slices (i.e., each of the groups x time), showed that only the Pred + sham group did not show significant recovery. That is, each of the other treatment groups showed a significant F value for this measure ($F's[1,36] \geq 5.15$, $p's < .03$).

Biochemical Results Task 2, study 2.

Biomarker analysis was also determined from animals tested on the elevated plus maze (EPM). GFAP measurements in all serum samples were below the assay detection limit (data not shown), indicating that GFAP was not detectable 72 h after predator or BOP exposure. In contrast, phosphorylated Tau (pTau) and total Tau (Tau) showed significant changes in abundance in biofluids after treatment. PTau was decreased from 0.07 +/ - 0.01 U/well in predator + sham BOP (PS) compared to 0.15 +/- 0.05 U/well in sham predator + sham BOP (SS) (two-tailed t-test, $p \leq 0.05$). The change indicated a decrease of nearly 44% in PS treated animals (Figure 6 top panel). Tau was relatively unchanged in the treatment groups (Figure 6 middle panel). However, due to the decrease in pTau, the ratio of pTau to Tau was also significantly decreased after predator exposure. Tau was 0.67 +/- 0.20 after PS compared to 1.37 +/- 0.25 after SS alone, which reflected nearly a 49% decrease (two-tailed t-test, $p \leq 0.05$) (Figure 6 bottom panel).

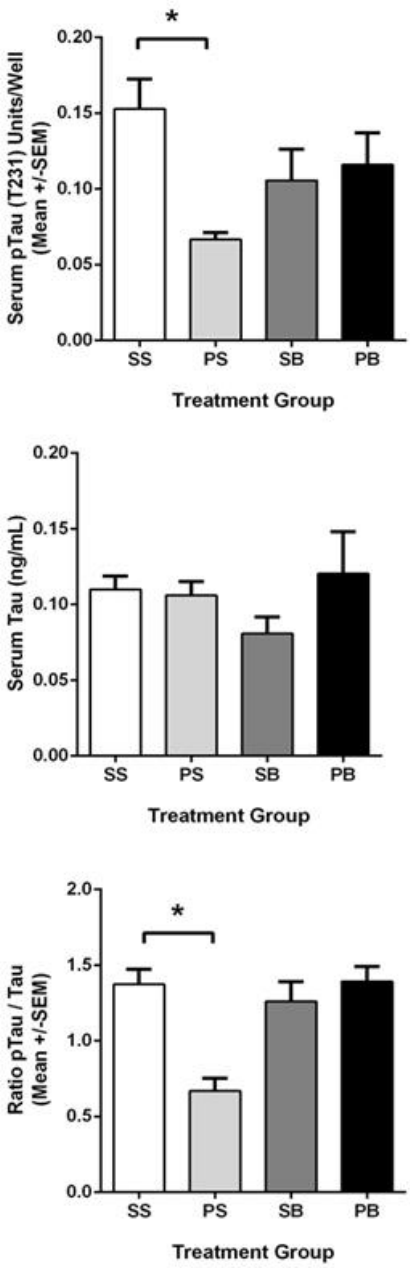

Figure 6. Quantitative ELISA of Tau and phospho-Tau in serum after predator exposure and / or BOP treatments. Serum samples are from rats in Experiment 2 and were sacrificed 72 h following the last BOP exposure and EPM analysis. Quantitation of serum phospho-Tau (upper panel), Total Tau, (middle panel) and the ratio phospho-Tau/total Tau (lower panel). Data is shown as the mean +/- SEM (N = 5/group). Asterisks indicate $p \leq 0.05$, two-tailed t-test.

Discussion and Conclusion, Task 2, study 2.

The results of the study are difficult to interpret because all treatment conditions, including the sham-sham treatment, produced a significant decrease in performance on the EPM when tested 24 h after the last BOP or predator exposure. In previous

implementations of the predator exposure, sham procedures have failed to produce a significant change in EPM performance (e.g., Genovese et al., 2014). A difference between previous implementations of the predator exposure and the instant study, however, is that all treatment groups received anesthesia (5% isoflurane x 3) as part of the BOP or sham-BOP treatment. It is possible that the anesthesia administered during the three daily treatments had residual effects that were seen 24 h later as a decrease in exploratory behavior. This explanation is supported by the observation that treatment groups not receiving any predator exposures (sham + BOP and sham + sham), showed a complete, or nearly complete recovery from the exploratory deficit when tested at the 72 h time point. That is, performance on the EPM recovered to baseline levels. It is notable that the Predator + sham group did not show a significant recovery – a result consistent with the relative persistent effects of predator exposures observed previously (Genovese et al., 2014). In this regard, the Predator + BOP treatment group showed some partial recovery at 72 h, but less than groups not receiving predator exposure. Unfortunately, the differences between groups were not statistically significant and preclude forming other conclusions.

Increases in tau and pTau have been reported in models and clinical samples of TBI (Rubenstein et al., 2014). This observed decrease in pTau 24 h after treatments was initially confounding. However, pTau has been negatively associated with increased arterial blood pressure (Glodzik et al., 2014) which may be occurring after predator exposure (Dielenberg, Carrive, & McGregor, 2001; Glodzik et al., 2014; Olsson & Hydbring-Sandberg, 2011). The decrease in pTau may be linked to increased stress in this model of predator exposure if in fact stress leads to changes in arterial pressure. However, this data is quite novel and rationale is currently speculative.

Task 3: Characterization of BOP on conditioned fear with subsequent biomarker evaluation

Task 3a, 8 week duration.

To address the issue of whether mTBI can modify a conditioned fear, we designed and implemented a conditioned fear procedure that is embedded in an operant behavior task. That is, rats are first trained to lever-press for food reinforcement under a variable-interval 32 second schedule of food reinforcement (VI32). The schedule produces a relatively constant rate of responding (lever pressing) throughout the 30 min test sessions. Once VI32 performance is stable, inescapable electric shock (IES or Unconditioned Stimulus [US]) is paired with a conditioned stimulus (CS) consisting of flashing lights and a pulsing tone. The pairings take place in a different chamber as does the VI32 task and can reasonably be considered to constitute a different environmental context. Subsequently, the CS is presented during the VI32; one

presentation every 7 days after initial CS+US pairing, for a 56-day period (i.e., 8 weekly presentations). Subsequently, subjects are sacrificed for tissue harvesting and biomarker evaluation.

Four treatment groups were used (illustrated in the table below): IES (CS+US pairings) + BOP, sham IES (CS only) + BOP, IES (CS+US pairings) + sham BOP, and Sham IES (CS only) + sham BOP.

Table 3 Treatment conditions for Task 3a.

Group	Conditioning	mTBI	n
IES + sham (I-S)	CS + IES	sham	10
IES + BOP (I-B)	CS + IES	BOP	10
sham + BOP (S-B)	CS only	BOP	10
sham + sham (S-S)	CS only	sham	10

Figure 7 below illustrates the design of the study. Appendix 2, Methods and Procedures provides further details for the procedures and treatment conditions.

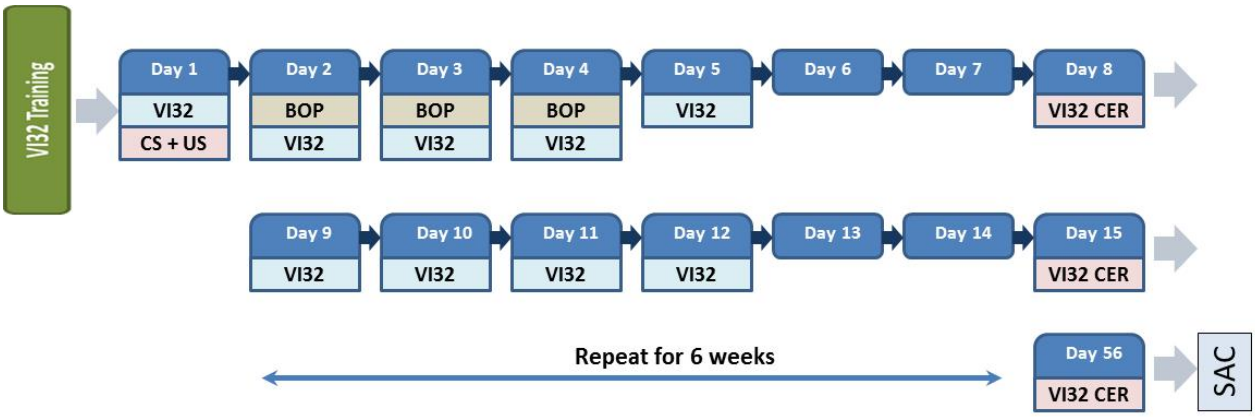

Figure 7. Experimental design for Task 3a

The association through pairing of the CS with the IES (or unconditioned stimulus, US) produces a conditioned fear. The impact or strength of the conditioned fear is evidenced by a suppression of responding on the VI32 task when the CS is presented (i.e., the conditioned response [CR] of conditioned suppression). We quantify the impact of the conditioned fear by calculating a suppression index according to the formula: (response rate before the CS - response rate after the CS) / (response rate before the CS + response rate after the CS). We typically calculate index values for intervals of 1 and 3 minutes before and after presentation of the CS. Additionally, we calculate the amount

of time that passes following the end of the presentation of the CS (or a few seconds after the end of the CS to eliminate scoring lever presses that might be in progress at the time of the CS) until the rat engages in another lever press. This “pause time” measure is clearly not independent of the aforementioned suppression index, but might represent a separate process or otherwise be an informative supplementary measure of the impact of the conditioned fear.

We contend that our implementation of a conditioned fear model is superior to alternative models that evaluate the strength of the conditioned fear only through a cessation of spontaneous motor activity (i.e., freezing). The advantage is that the current model assesses the impact relative to the disruption of a learned and highly motivated behavior in contrast to a disruption of an unlearned and nonspecific behavior (spontaneous motor activity). Thus, we believe that results from the approach are likely to be more relevant for the study of the quality of life impact of conditioned emotional responses related to aversive and traumatic experiences.
To evaluate the impact of BOP on conditioned fear, our initial data analysis strategy is to perform a two factor ANOVA (4 treatments X 8 time points [repeated]). ANOVA and contrasts were performed using SAS analytical software (Proc Mixed) and a Satterthwaite approximation for the denominator degrees of freedom was employed. Several covariance structures were tested for fit using AIC and BIC measures resulting in the choice of an autoregressive covariance structure for the model (Kincaid, 2005; Littell et al., 2000; Wolfinger & Chang, 1995). Probability values < .05 were treated as statistically significant.

Behavioral Results Task 3a.

In addition to the results and discussion below, we include a publication containing Task 3a in the appendix.

No grossly observable effects from the IES or from BOP exposures (excepting the time for recovery from anesthesia) were noted. That is, all rats appeared normal during weighing and handling throughout the experiment.

Responding under the VI schedule was acquired by all rats. Baseline measures of responding on the active lever (i.e., the lever producing food reinforcement), defined as the average of the last 6 sessions conducted before exposure, for the treatment groups (n=10 each group) were as follows (mean ± SEM responses per min): I-B=56.5 ± 10.3, I-S=51.4 ± 6.9, S-B=50.1 ± 3.7, S-S=50.7 ± 5.1. Figure 8 shows performance on the VI from the last baseline session through the first CR test. ANOVA evaluating VI performance during the five sessions after fear conditioning and including the session

with the first CR test revealed no significant effects for group ($F[3,36]=0.96$, $p>.05$) or the group by session interaction ($F[12,144]=0.75$, $p>.05$) but did reveal a significant main effect for session ($F[4,144]=8.71$, $p<.001$). Tests of effect slices for the session factor showed significant effects for the I-B ($F[4,144]=3.32$, $p<.01$) and S-S ($F[4,144]=3.96$, $p<.01$) treatment groups but not for the I-S ($F[4,144]=1.38$, $p>.05$) or S-B ($F[4,144]=2.31$, $p>.05$) groups. Although there was not a significant main effect for groups, we were particularly interested in whether any changes could be attributed to a common treatment of IES or BOP presentation. Thus, we evaluated, but found no significant effects for, contrasts comparing groups receiving BOP (I-B and S-B) vs. no BOP (I-S and S-S) ($F[1,36]=1.83$, $p>.05$), and IES (I-B and I-S) vs. no IES (S-B and S-S) ($F[1,36]=1.06$, $p>.05$).

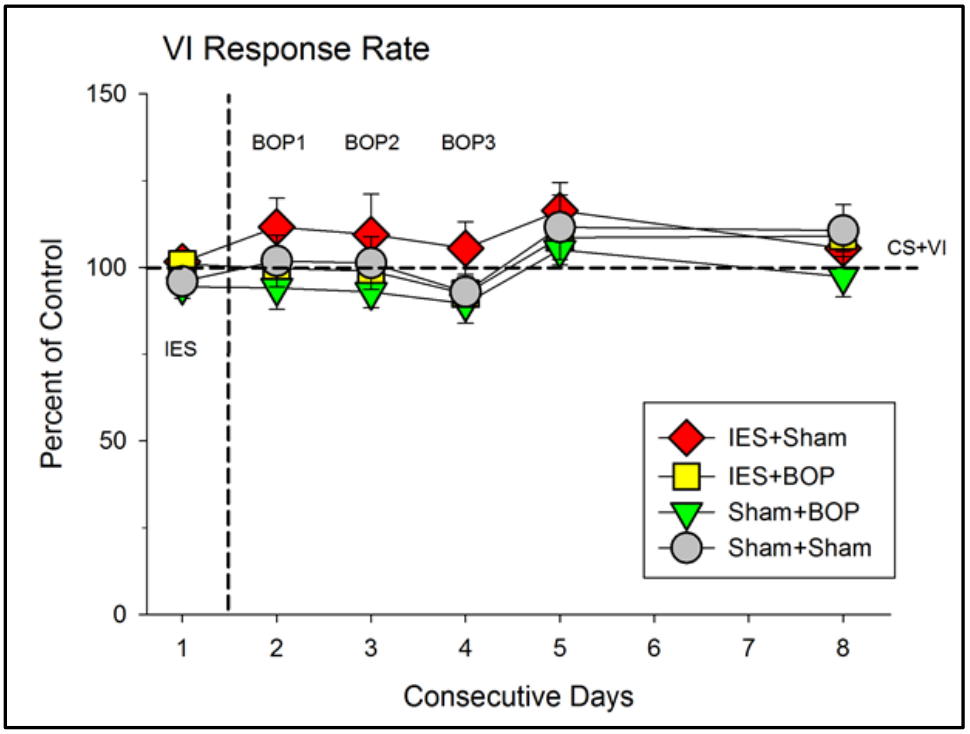

Figure 8. Performance on the VI schedule of reinforcement during six consecutive test sessions. CS + IES (or sham) occurred following the test session on day 1. BOP (or sham) occurred ~2 h before the test sessions on days 2-4. The CS alone was presented during the session on day 8. Ordinate: Response rate as a percentage of control (determined as the average response rate from six baseline sessions). Abscissa: Consecutive days. Each point represents the mean (+/- SEM) from 10 rats. Dashed horizontal line indicates control rate of responding. Points to the left of the vertical dashed line represent the last baseline session.

Figure 9 shows VI performance over eight weeks beginning with the session following CS+IES (or CS+sham-IES). In general, performance on the VI was maintained near baseline levels in all groups, although some deviations from baseline were present.

ANOVA showed no main effects for groups ($F[3,36]=1.77$, $p>.05$) and no groups by session interaction ($F[21,252]=1.21$, $p>.05$), but did show a significant main effect for sessions ($F[7,252]=2.07$, $p<.05$). Analysis of effect slices for sessions revealed a significant effect only for the I-S group ($F[7,252]=2.44$, $p<.05$). No significant effect was found for contrasts that compared IES groups vs. no IES groups (I-S and I-B vs. S-B and S-S, $F[1,18]=.03$, $p>.05$) or BOP groups vs. no BOP groups (I-B and S-B vs. I-S and S-S, $F[1,18]=5.03$, $p>.05$).

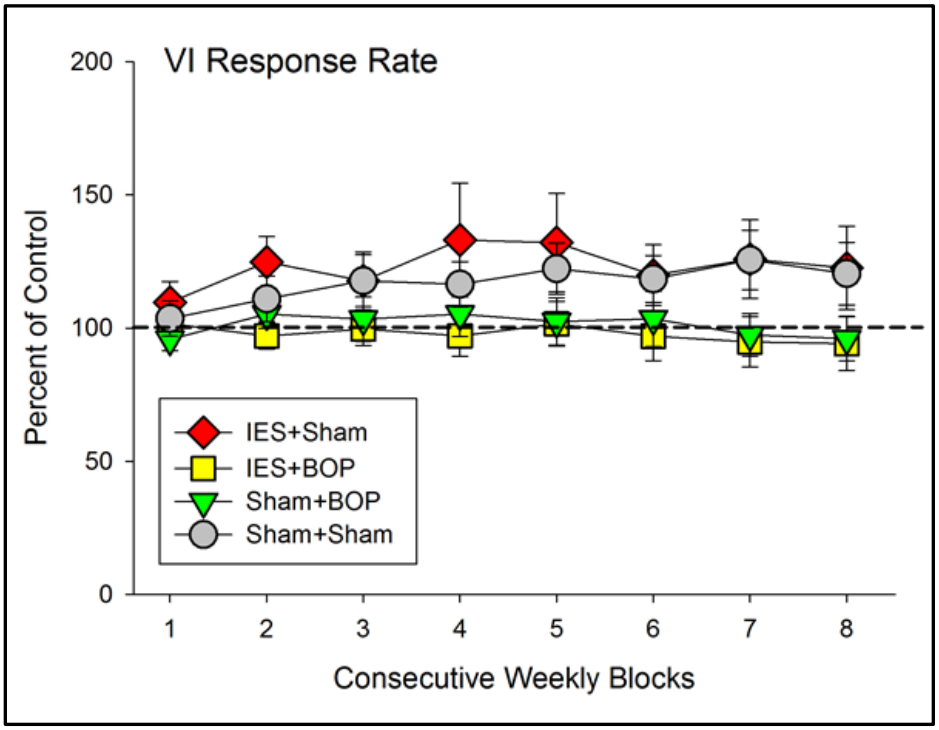

Figure 9. Performance on the VI schedule during eight consecutive weeks. Ordinate: Response rate as a percentage of control (determined as the average response rate from six consecutive baseline sessions). Abscissa: Consecutive blocks. Each point represents the mean (+/- SEM) from 10 rats and each block contains the average response rate from five sessions. Dashed horizontal line indicates control rate of responding.

Figure 10 presents the extinction functions for conditioned fear, as evidenced by the degree of response suppression (suppression index for +/- 1 min [top] and +/- 3 min [bottom]), for the four treatment groups during the eight consecutive weekly CR tests. As expected, presentation of the CS during the VI session initially produced substantial response suppression in treatment groups where the CS had been previously paired with IES (i.e., I-S and I-B). Also as expected, the CS produced very little response suppression in treatment groups where the CS had not been paired with IES. In general, when present, the response suppression produced by the CS was greater for the +/- 1 min index as compared with the +/- 3 min index. For the +/- 1 min suppression index (Fig. 10, top panel), ANOVA showed a significant main effect for treatment group

(F[3,82.6]=22.62, p<.001), CR session (F[7,180]=10.22, p<.001) and the treatment group by CR session interaction (F[21,180]=2.87, p<.001). Similarly, for the +/- 3 min suppression index (Fig. 10, bottom panel), ANOVA showed a significant main effect for treatment group (F[3,61.3]=9.95, p<.001), CR session (F[7,180]=6.82, p<.001) and the treatment group by CR session interaction (F[21,180]=3.78, p<.001). For both the I-S and I-B treatment groups, the conditioned fear diminished with continued presentation of the CS as can be seen by a reduction in both suppression indices during the later CR test sessions. Analyses of the effect slices for CR sessions for the +/- 1 min suppression index showed significant effects for both the I-S (F[7,180]=11.39, p<.001) and I-B (F[7,180]=6.80, p<.001), but not for the S-B (F[7,180]=.31, p>.05) and S-S (F[7,180]=.034, p>.05). The same profile of significance was found for the +/- 3 min suppression index (I-S, F[7,180]=14.82, p<.001; I-B, F[7,180]=2.66, p<.02; S-B, F[7,180]=.49, p>.05; S-S, F[7,180]=.17, p>.05). As can be seen from Figure 10, conditioned fear in the I-S group was, on average, greater than that in the I-B group. Contrasts between these two groups across CR sessions showed a significant difference for both the +/- 1 min index (F[1,82.6]=9.24, p<.005) and the +/- 3 min index (F[1,61.3]=6.42, p<.02). The difference between these two groups is also illustrated in Figure 11 which shows the degree of suppression (suppression index for +/- 1 min [top] and +/- 3 min [bottom]) for the four treatment groups during the first CR test. Contrasts comparing the treatment groups at this time point show a significant difference in suppression between the I-S and I-B treatments for both the +/- 1 min (top panel, F[1,268]=7.54, p<.01) and the +/- 3 min (bottom panel, F[1,223]=26.54, p<.001) indices. Additionally, both the I-S and I-B groups were significantly different than both the S-B and S-S groups for the +/- 1 min (Fs[1,223]≥21.48, ps<.001) and the +/- 3 min (Fs≥10.75, ps<.01) indices. The S-B and S-S groups did not differ significantly for either index (+/- 1 min index, F[1,268]=.22, p>.05; +/- 3 min index, F[1,223]=.30, p>.05).

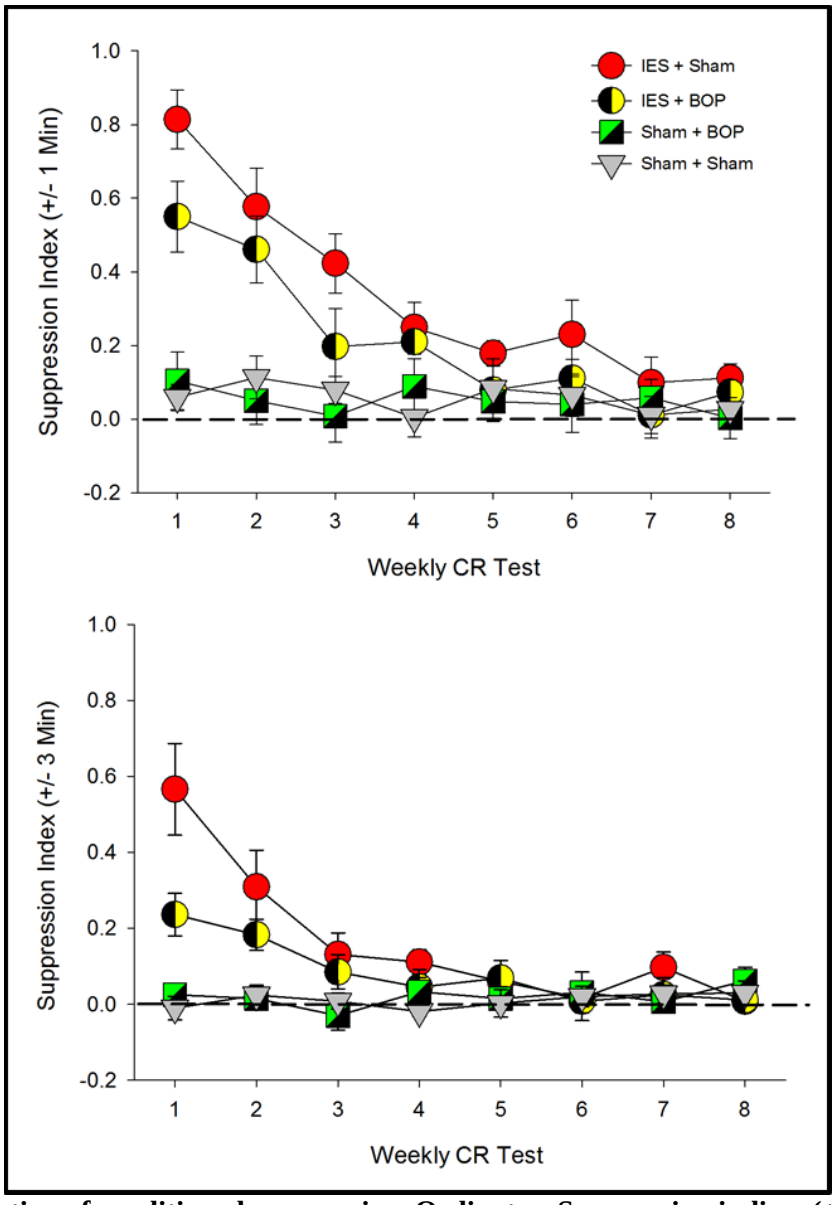

Figure 10. Extinction of conditioned suppression. Ordinates: Suppression indices (+/-1 min, top and +/-3 min, bottom) during eight weekly CR test sessions. Abscissas: Consecutive weeks. Each point represents the means (+/- SEM) from 10 rats. Dashed horizontal lines represent a suppression index value of 0 indicating the same rate of responding before the CS as after the CS (i.e., no response suppression).

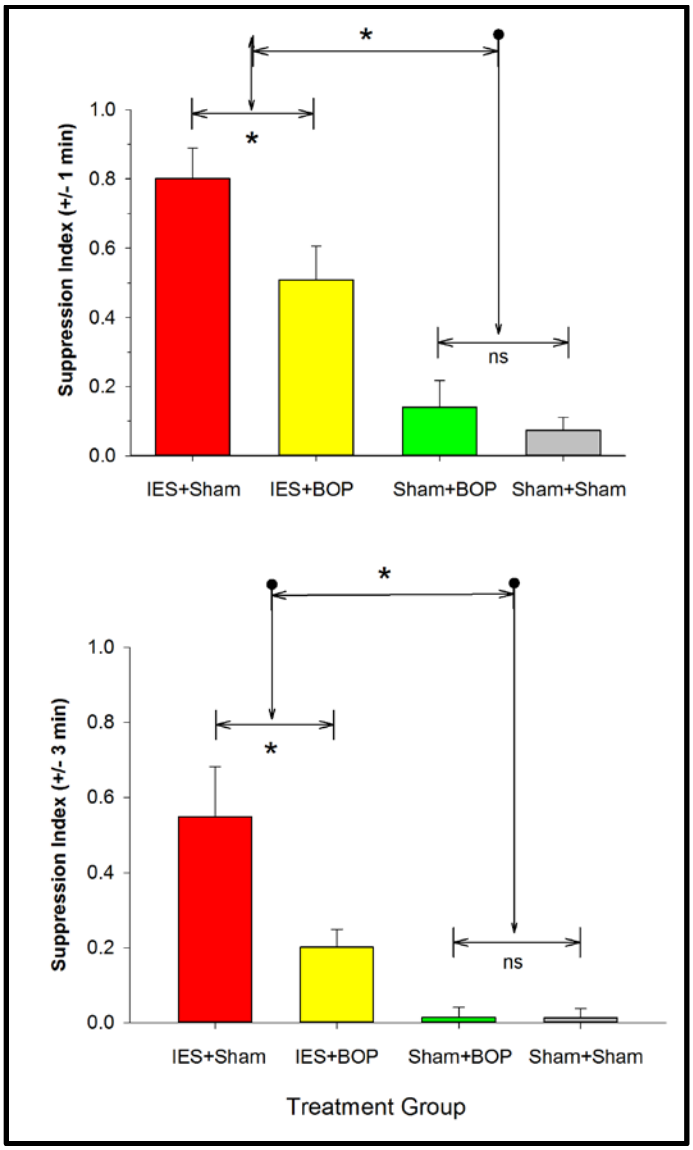

Figure 11. Conditioned suppression during the first CR test administered seven days after CS + IES pairing and four days after the last BOP exposure. Ordinates: Suppression indices (+/-1 min, top and +/-3 min, bottom). Abscissas: Four treatment groups: IES + sham-BOP (I-S), IES + BOP (I-B), sham-IES + BOP (S-B) and sham-IES + sham-BOP (S-S). Bars represents the mean (+SEM) from 10 rats. Asterisks indicate a statistically significant difference (contrasts following ANOVA, ps<.05) and "ns" indicates comparison not statistically significant.

Biochemical Results Task 3a.

This study was continued in order to determine the specific response of the most promising individual TBI biomarker proteins candidates during a chronic post-treatment time point. Several brain regions (prefrontal cortex (PFC), cortex (CTX), midbrain (M), hippocampus (HP) and cerebellum (CB)), were dissected from sham IES + sham BOP (SS), IES + sham BOP (IS), sham IES + BOP (SB), and IES + BOP (IB) treatment

groups 8 weeks after the last BOP. The relative abundance of GFAP, UCH-L1, PSD-95, nNOS, MBP, as well as α-II spectrin and its break down products (BDPs) was determined in each brain region and treatment group. All proteins were analyzed by semi-quantitative western blotting and densitometry or quantitative ELISA as indicated. Data shown reflect the relative abundance of each protein after normalization to the value detected in sham IES + sham BOP (SS) controls. Therefore, SS values are equal to “1“ for each brain region.

Relative abundance of GFAP was not altered by any treatment (Figure 12, top panel). UCH-L1 abundance was altered in multiple brain regions. After SB, UCH-L1 was marginally increased in the PFC, although not significant from SS ($p = 0.07$). In contrast, a ~50 % increase of UCH-L1 to 1.50 +/- 0.13 (AU) within the CTX after IB was detected. In addition, a small increase to 1.18 +/- 0.02 (AU) was detected in the M after IS (Figure 12, bottom panel).

The relative abundance of synaptic density proteins, PSD95 and nNOS, was determined. PSD-95 was decreased in the CB by ~35% to 0.65 +/- 0.15 (AU) after IS, which was statistically significant from SS controls ($p \leq 0.05$, two-tailed unpaired t-test) (Figure 13, top panel). NNOS decreased by ~23% to 0.78 +/- 0.08 (AU) after IS treatment compared to SS ($p \leq 0.05$, two-tailed unpaired t-test). SB also lead to a decrease in NNOS, but this change was not significant compared to SS ($p = 0.09$) (Figure 13, bottom panel). MBP was generally unaffected by IS, SB, and IB treatments. In the midbrain (M), MBP was increased after IS and IB, however the results were not significant from SS (Figure 14).

Next, the relative abundance of full length α-II spectrin (detectable at 280kDa) was compared to generation of its 145/150 and 120 kDa break-down products (SBDPs). Each brain region was analyzed individually and normalized to the densitometric value of α-II spectrin in SS treated cohorts. Therefore, the value SS of 280kDa was equivalent to “1” +/- SEM. Thereafter, α-II spectrin (280kDa) or SBDPs were each compared to respective values detected in SS for each molecular weight studied (Figure 15). There was no significant increase in SBDP145/150 in the PFC after any treatment compared to SS. In the CTX, IB led to a loss in α-II spectrin (280kDa). The relative mean densitometry was 0.36 +/- 0.15 (AU) and represented a 64% decrease compared to SS ($p \leq 0.05$, two-tailed unpaired t-test). There was no difference in SBDP-145/150 after IS, SB, or IB treatment. With the exception of a very small increase in CTX after IB (not significant from SS), SBDP-120 was not detectable (Figure 15, panel B). Alpha-II spectrin (280kDa) and its BDPs were not changed in the midbrain (M), hippocampus (HP), or cerebellum (CB).

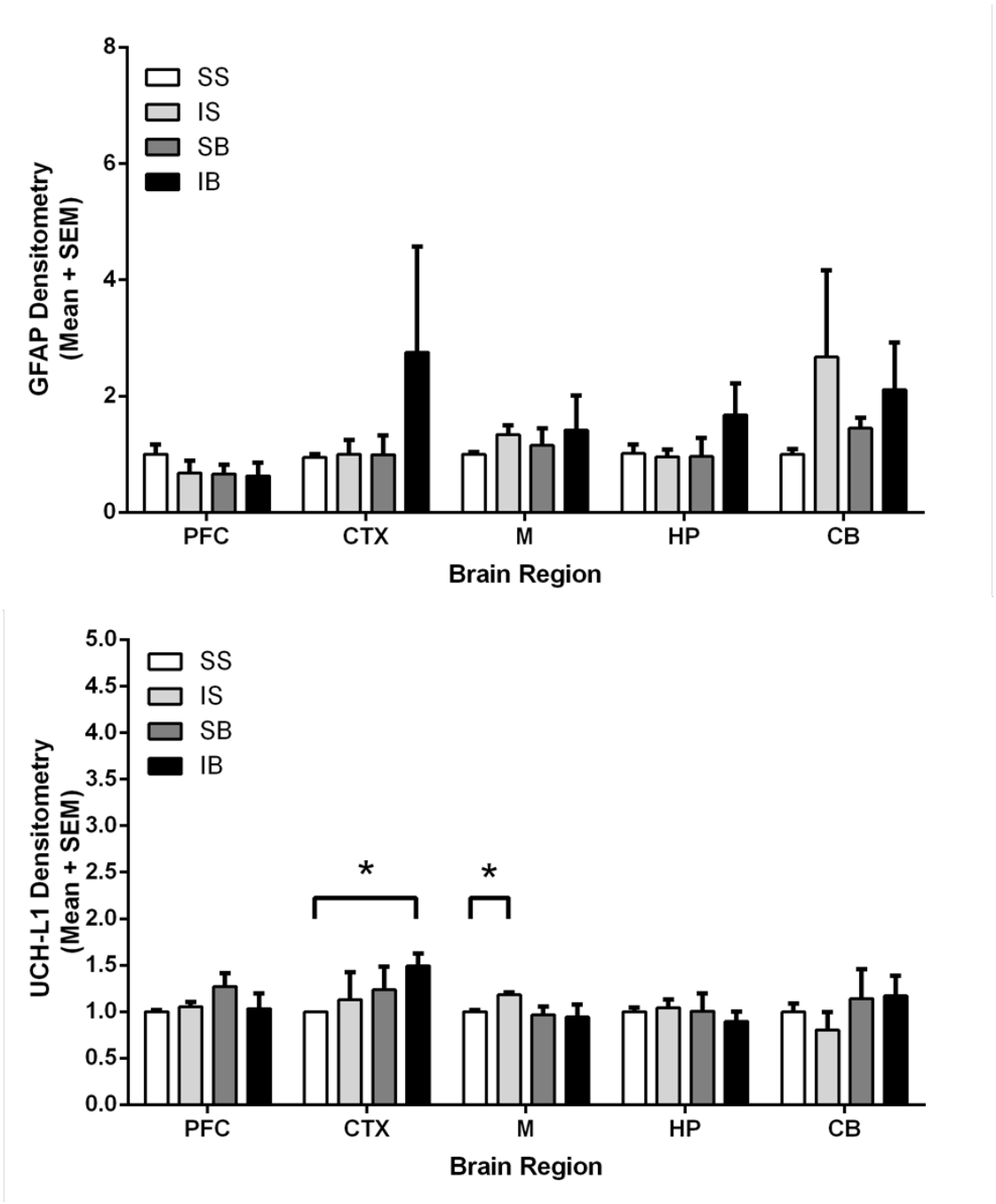

Figure 12. Semi-quantitative Western blotting of GFAP (top) and UCH-L1 (bottom) after fear conditioning (IES) and mTBI (BOP). Tissue samples are from rats in Task 3a and were sacrificed 8 weeks following the last BOP exposure. Densitometry was individually measured in clarified tissue lysates from specific brain regions: prefrontal cortex (PFC), cerebral cortex (CTX), hippocampus (H) and cerebellum (CB). The relative fold change of protein band densitometry quantitation is shown as the average +/- SEM (N = 5-6/group).

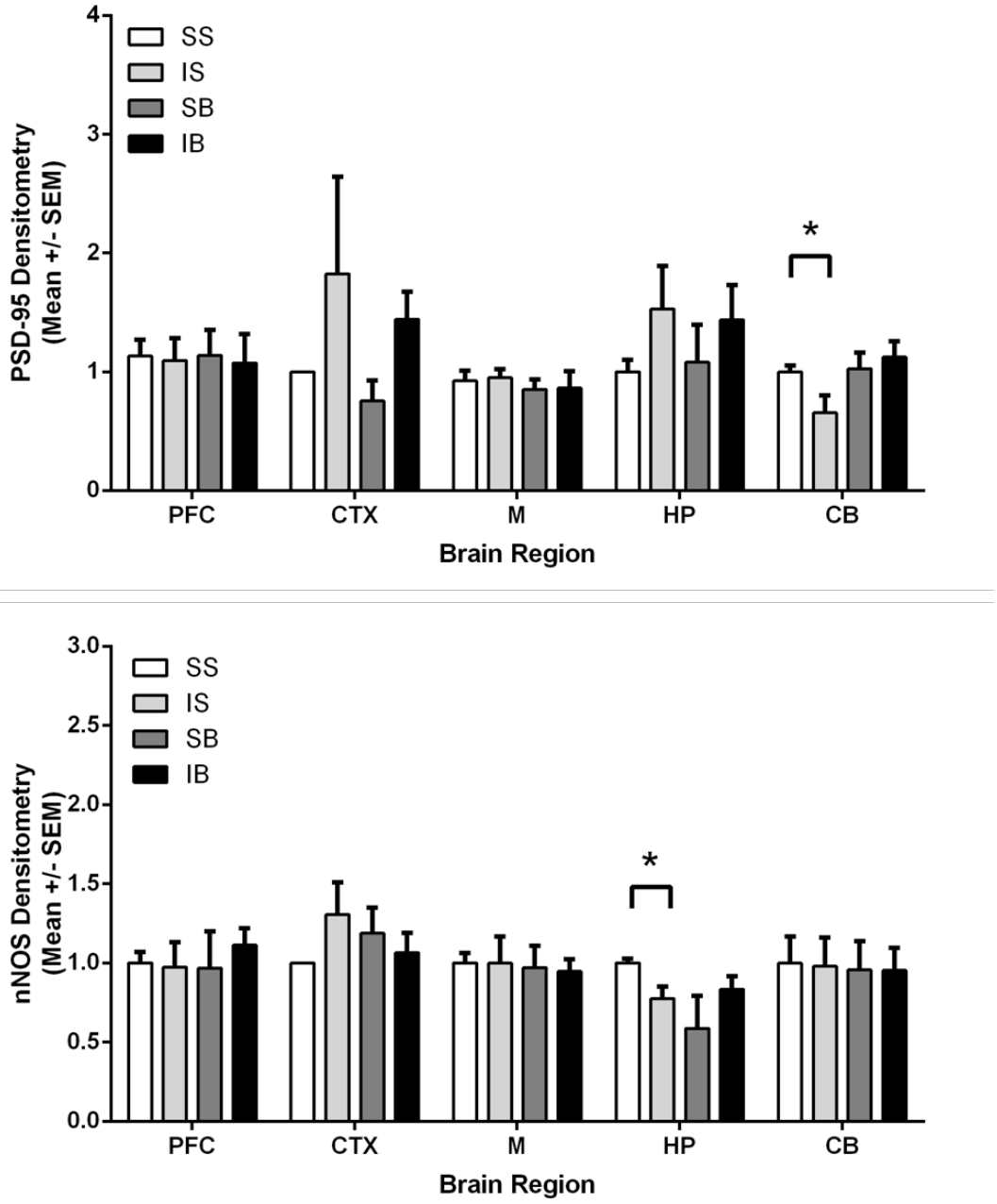

Figure 13. Semi-quantitative Western blotting of PSD-95 (top) and NNOS (bottom) after fear conditioning (IES) and mTBI (BOP). Tissue samples are from rats in Task 3a and were sacrificed 8 weeks following the last BOP exposure. Densitometry was individually measured in clarified tissue lysates from specific brain regions: prefrontal cortex (PFC), cerebral cortex (CTX), hippocampus (H) and cerebellum (CB). The relative fold change of protein band densitometry quantitation is shown as the average +/- SEM (N = 5-6/group).

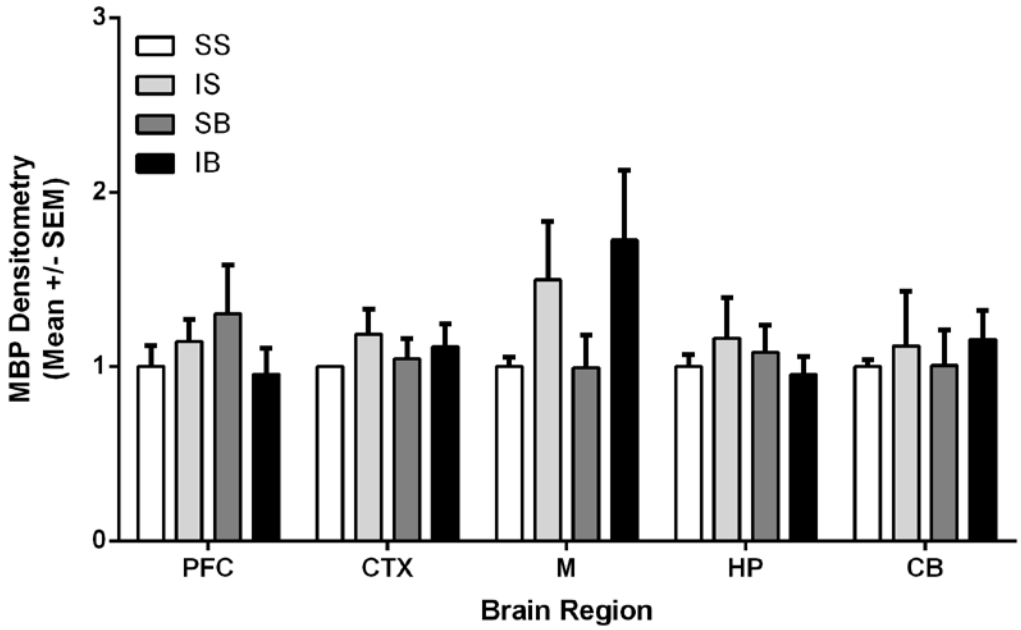

Figure 14. Semi-quantitative Western blotting of MBP after fear conditioning (IES) and mTBI (BOP). Tissue samples are from rats in Task 3a and were sacrificed 8 weeks following the last BOP exposure. Densitometry was individually measured in clarified tissue lysates from specific brain regions: prefrontal cortex (PFC), cerebral cortex (CTX), hippocampus (H) and cerebellum (CB). The relative fold change of protein band densitometry quantitation is shown as the average +/- SEM (N = 5-6/group).

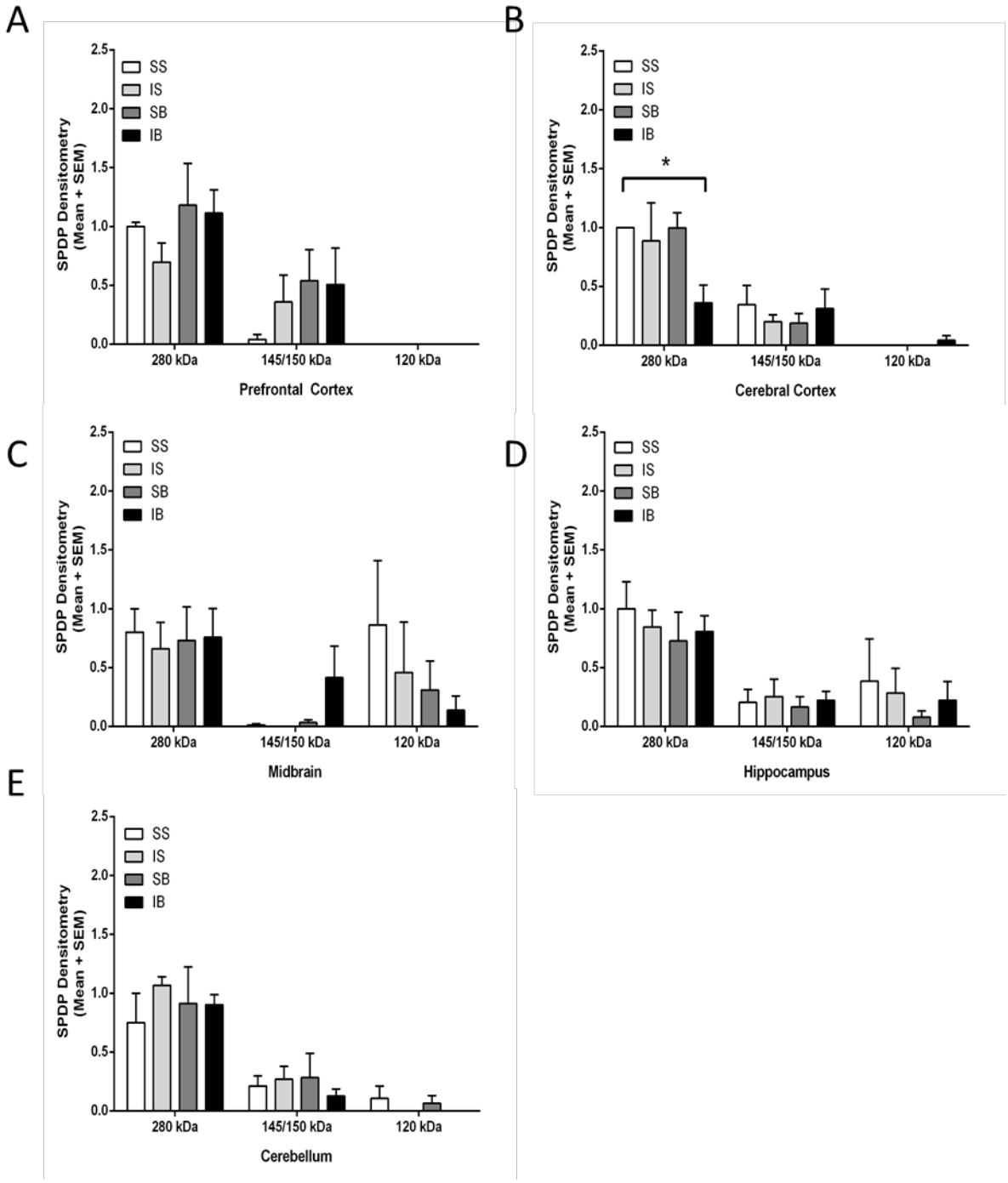

Figure 15. Alpha-II spectrin and its break down products (BDPs). Semi-quantitative Western blotting of α-II spectrin (280kDa) and SBDPs (145/150 and 120 kDa) after fear conditioning (IES) and mTBI (BOP). Tissue samples are from rats in Task 3a and were sacrificed 8 weeks following the last BOP exposure. Densitometry was individually measured in clarified tissue lysates from specific brain regions: prefrontal cortex (A), cerebral cortex (B), midbrain (C), hippocampus (D) and cerebellum (E). The relative fold change of protein band densitometry quantitation is shown as the average +/- SEM (N = 5-6/group).

Discussion and Conclusion, Task 3a.

BOP reduced the degree of conditioned suppression. That is, as compared to sham-BOP controls, BOP decreased the expression of a conditioned fear that was trained prior to exposure. There are several possible interpretations of this result. First, it could be argued that the BOP produced sensory damage to the auditory and/or visual system such that the perception of the CS was altered in exposed rats. While BOP, using a similar procedure as in the present study, has been reported to produce visual system degeneration, it did so only at substantially higher pressures (104-173 kPa) and a pressure of 84 kPa, which is greater than that used in the present study, did not result in any visual system pathology (Petras et al., 1997). Additionally, the exact regimen of BOP used in the present study was not found to produce any changes in the prepulse inhibition of a startle response, suggesting that auditory perception was also not impaired (Elder et al., 2012). Therefore, it is not likely that sensory damage due to BOP was responsible for the observed difference between the I-S and I-B treatment groups.

A second interpretation of this result is that the BOP produced a retrograde amnesia. In this regard, it is notable that the BOP exposures took place beginning at ~22 hours after the IES. It is likely that enough time had elapsed for memory consolidation of the event to have occurred (McGaugh, 2000). Thus, the amnesic effect would not have been through the disruption of memory consolidation processes such as when the insult takes place shortly after the conditioning event. Furthermore, a single BOP at the same and at a greater pressure than used in the present study did not produce an amnesic effect when exposure immediately followed a passive avoidance task (Ahlers et al., 2012). Typically, more severe injuries are required to produce a retrograde amnesia for events already presumed to be consolidated into long term memory (e.g., Chen et al., 2009). It is also notable that BOP exposed animals did show a conditioned fear, although to a lesser degree than the sham-BOP treatment group. Thus, the retrograde amnesia would have to be characterized as partial.

While a retrograde amnesia cannot be completely ruled out, we propose that the BOP exposure more likely decreased behavioral inhibition. That is, responding on the VI task is maintained by food reinforcement and the schedule of reinforcement exerts a degree of stimulus control (i.e., represents a motivated task). Following pairing with the IES, the CS elicits a conditioned response (i.e., conditioned fear) which is in conflict with responding on the VI task. In this sense, the CS serves as an inhibitory or “stop” signal. The BOP exposure appears to have decreased the inhibitory control exerted by the CS although responding on the VI was unaffected. While further studies are needed to confirm this possibility, it is notable that failures of inhibitory control behaviors are integral features of many psychiatric disorders and the mechanism of an inhibitory

control system in rats has been the subject of substantial study (see review by Eagle & Baunez, 2010).

The decreased expression of a conditioned fear produced by BOP in the present study represents a functional deficit. That is, the optimal conditioned fear response is best represented by the IES+sham-BOP treatment and a substantial deviation from that response can reasonably be interpreted as an adverse outcome.

There was a statistically significant difference in the extinction functions between the two groups, but that difference is consistent with the decreased initial conditioned fear response produced by BOP. Furthermore, both groups reached near zero values for the suppression indices that were equivalent to groups that had not received CS+IES pairing. We, therefore, conclude that BOP did not delay or facilitate extinction to a conditioned fear, although it did alter the magnitude of its expression.

Targeted biomarkers: UCH-L1 (a deubiquitinating enzyme) is a key component of the proteosome pathway and is a biomarker of neuronal loss in a mild closed head TBI model (Chen et al., 2012). UCH-L1 was increased 8 weeks after IB in the cortex and after IS in the midbrain. On one hand, this increase may signify increased protein stability in the form of aggregates often detected in models of age-related neurodegeneration (Proctor, Tangeman & Ardley, 2010). On the other, increased UCH-L1 may identify a novel aspect of synaptic density damage as UCH-L1 and downstream pathways affect synaptic function (Mabb & Ehlers, 2010). Although there are many potential reasons for increased UCH-L1, the latter (synaptic function) may be more likely, since Cerebellar PSD-95 and hippocampal nNOS loss was detectable at 8 weeks after IS treatment. Overall synaptic proteins were negatively affected by treatment and loss of dendrites at this time point is likely as it has been reported in a murine model (Sanders, Cowansage, Baumgartel, & Mayford, 2012).

Task 3b, 24 h duration.

Task 3b was implemented using identical procedures as task 3a except that 1) Only a single CER test was conducted, and 2) the CER test took place ~24 h after the last BOP exposure. The group size was decreased and the emphasis was on the detection of large biochemical changes, rather than behavioral changes. Since Task 3a had a sacrifice time point ~ 8 weeks after treatments, it was conceivable that short-term biochemical changes might not be observed that long after treatment.

The same four treatment groups as were used in Task 3a were also used in Task 3b (illustrated in the table below and included: IES (CS+US pairings) + BOP, sham IES

(CS only) + BOP, IES (CS+US pairings) + sham BOP, and Sham IES (CS only) + sham BOP.

Table 4 Treatment conditions for Task 3b.

Group	Conditioning	mTBI	N
IES + sham (I-S)	CS + IES	sham	5
IES + BOP (I-B)	CS + IES	BOP	5
sham + BOP (S-B)	CS only	BOP	5
sham + sham (S-S)	CS only	sham	5

The experimental design is illustrated in Figure 16 below.

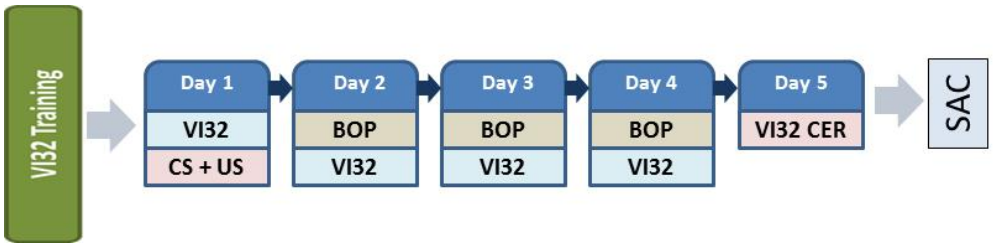

Figure 16 Experimental design for Task 3b

Behavioral Results Task 3b.

As in Task 3a, responding under the VI schedule was acquired by all rats. Baseline measures of responding on the active lever (i.e., the lever producing food reinforcement), defined as the average of the last 6 sessions conducted before exposure, for the treatment groups (n=5, each group) were as follows (mean ± SEM responses per min): I-B=71.1 ± 7.2, I-S=89.8 ± 9.8, S-B=50.2 ± 8.8, S-S=71.4 ± 9.4. In general, performance on the VI was maintained near baseline levels in all groups during the test sessions after BOP exposures and the CR test session, although some deviations from baseline were present. Figure 17 shows performance on the VI from the last baseline session through the first CR test. ANOVA evaluating VI performance during the four sessions after fear conditioning and including the session with the CR test revealed no significant effects for group ($F[3,16]=2.29$, $p>.05$), session ($F[3,48]=1.08$, $p>.05$) or the group by session interaction ($F[9,48]=0.42$, $p>.05$).

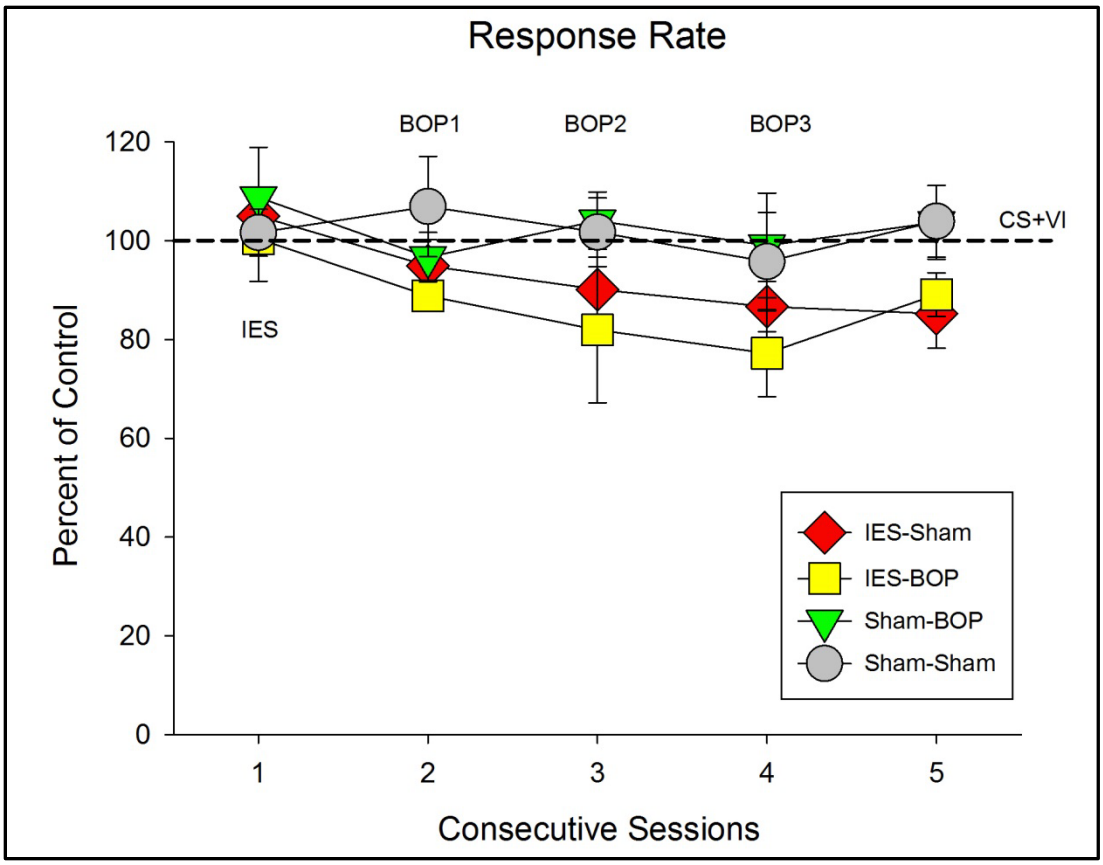

Figure 17. Performance on the VI schedule of reinforcement during five consecutive test sessions. CS + IES (or sham) occurred following the test session on day 1. BOP (or sham) occurred ~2 h before the test sessions on days 2-4. The CS alone was presented during the session on day 5. Ordinate: Response rate as a percentage of control (determined as the average response rate from six consecutive baseline sessions). Abscissa: Consecutive days. Each point represents the mean (+/- SEM) from 5 rats. Dashed horizontal line indicates control rate of responding. Points to the left of the vertical dashed line represent the last baseline session.

Figure 18 shows the degree of suppression (suppression index for +/- 1 min [top] and +/- 3 min [bottom]) for the four treatment groups during the CR test. ANOVA showed a significant difference for both the +/- 1 min (top panel, $F[3,15]=13.56$, $p<.001$) and the +/- 3 min index (bottom panel, $F[3,15]=5.74$, $p<.01$). As expected, and as observed in Experiment 1, presentation of the CS during the VI session produced substantial response suppression in treatment groups where the CS had been previously paired with IES (i.e., I-S and I-B). Also as expected and observed in Experiment 1, the CS produced very little response suppression in treatment groups where the CS had not been paired with IES. In this regard, multiple comparisons revealed significant differences in both suppression indices between the I-S or I-B groups and either the S-B or S-S groups (Fisher's t, $ps<.05$). Unlike in Experiment 1, however, the degree of suppression was equivalent between the I-S and I-B groups and multiple comparisons revealed no significant difference between the I-S and I-B groups for either the +/- 1 min (Figure 18, top panel) or +/- 3 min (Figure 18, bottom panel) indices (Fisher's t, $ps>.05$).

No difference was found between the S-B and S-S groups for either index (Fisher's t, ps>.05).

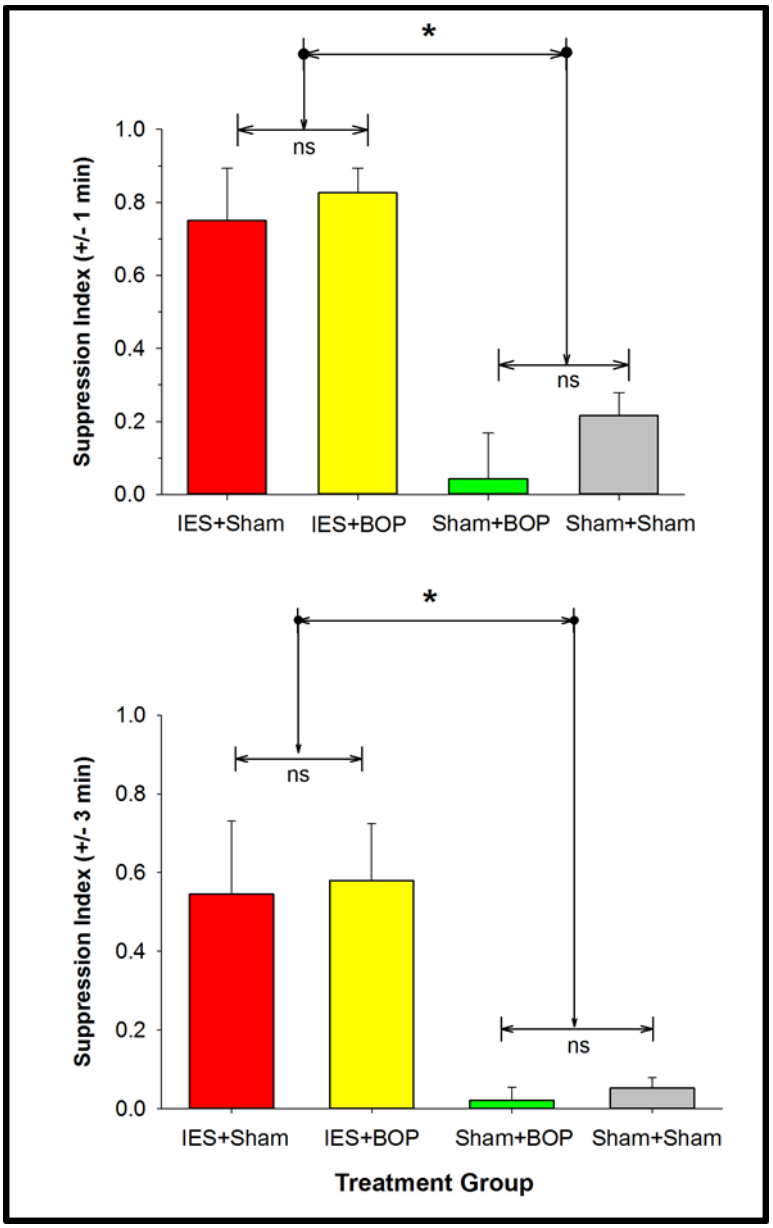

Figure 18. Conditioned suppression during the first CR test administered four days after CS + IES pairing and ~26 h after the last BOP exposure. Ordinates: Suppression indices (+/-1 min, top and +/-3 min, bottom). Abscissas: Four treatment groups: IES + sham-BOP (I-S), IES + BOP (I-B), sham-IES + BOP (S-B) and sham-IES + sham-BOP (S-S). Bars represents the mean (+SEM) from 5 rats. Asterisks indicate a statistically significant difference (Fisher's LSD following ANOVA, ps<.05) and "ns" indicates comparison not statistically significant.

Biochemical Results Task 3b.

This study determined the specific response of individual protein biomarkers with spatial-temporal resolution in animals treated with either inescapable electric shock (IES) and/or blast overpressure (BOP). Several brain regions (prefrontal cortex (PFC), cortex (CTX), midbrain (M), hippocampus (HP) and cerebellum (CB)), were dissected from each of the 4 treatment groups at 24 h after the last BOP event. The experimental groups were sham IES + sham BOP (SS), IES + sham BOP (IS), sham IES + BOP (SB) and IES + BOP (IB). As with studies conducted 8 weeks after treatment, multiple key TBI related proteins were included within analyses: glial fibrillary acidic protein (GFAP) , ubiquitin carboxy terminal hydrolase (UCH)-L1, syntaxin-6 (syn-6), endothelial monocyte-activating polypeptide-II (p43/pro-EMAP-II), postsynaptic density protein (PSD)-95, neuronal nitric oxide synthase (nNOS), as well as full length α-II spectrin (280k) and its break down products (BDPs, 145/150kDa or 120 kDa).

Other proteins, such as MAP-2 was initially evaluated. However, detection and resolution of isoforms was poor and not pursued further. All proteins were analyzed by semi-quantitative western blotting and densitometry or quantitative ELISA as indicated. Data shown reflect the relative abundance of each protein after normalization to the value detected in sham IES + sham BOP (SS) controls. Therefore, SS values are equal to “1“ for each brain region.

Relative abundance of GFAP and UCH-L1 were unchanged in all treatment groups (Figure 19). Syntaxin-6 was decreased to 0.75 +/- 0.05 (AU) in the CB after IS (vs. SS, two-tailed t-test, $p \leq 0.05$) (Figure 20). P43/EMAP-II protein showed a sharp increase in abundance after SB in the PFC, but it was not significant. Relative abundance of p43/EMAP-II decreased in the M after SB to 0.62 +/- 0.10 (AU), a 39% decrease compared to SS (two-tailed t-test, $p \leq 0.05$) (Figure 21).

Two proteins that are key to synaptic densities were also analyzed after each treatment. PSD-95 and nNOS both exhibited changes in proteins abundance in various brain regions. The densitometry of PSD-95 in the HP after IB was 0.64 +/- 0.05 (AU) and reflected an approximate loss of 36% compared to SS (two-tailed t-test, $p \leq 0.05$) (Figure 22, top panel). Similarly, the IB also indicated loss of nNOS in the HP, such that mean densitometric values were 0.71 +/- 0.08 (AU) or 29% less than SS controls (two-tailed t-test, $p \leq 0.05$). NNOS was also decreased in the CB in this same treatment group, by nearly 36% compared to SS controls, to 0.64 +/- 0.07 (AU) (two-tailed t-test, $p \leq 0.05$) (Figure 22, bottom panel).

Analysis of the neuronal cytoskeletal protein, α-II spectrin (280kDa) and its BDPs (145/150kDa or 120 kDa), was conducted such that the full length protein (α-II spectrin (280kDa)) in SS was normalized to “1“ for each brain region. Therefore, relative

abundance of BDPs could be compared directly to full length α-II spectrin within the same samples (Figure 23). Overall, α-II spectrin, SBDP-145/150 and SBDP-120 were not significantly affected by treatments of IS, SB, or IB. Interestingly, the SBDP-120 fragment was clearly increased to 2.5 +/- 0.66 (AU) after IB in the PFC compared to SS ($p \leq 0.05$, 1-way ANOVA with Fisher's LSD post-test).

To further determine the effect of IS, SB, and IB on TBI biomarkers within biofluids, GFAP and UCH-L1 were measured in serum using the same cohorts for which brain tissue analysis was conducted. Serum GFAP was marginally increased in after IB treatment and UCH-L1 was moderately increased after SB treatment. However, both quantitative observations were not significantly different compared to serum isolated from SS treated animals (Figure 24 Serum GFAP top and UCH-L1 bottom). Analysis of CSF samples revealed no difference in GFAP after treatments compared to SS controls (not shown).

Discussion and Conclusion, Task 3b.

In Task 3a, rats were first tested for a conditioned fear beginning four days following the last BOP exposure. In study 3b, rats were tested for a conditioned fear only once, and the testing took place on the day following the last BOP exposure. Surprisingly, there was no difference in the expression of conditioned fear between BOP and sham-BOP treatments. While a smaller group size was used as compared to task 3a, the difference between the mean values for the suppression indices between the groups in task 3b is very small. It is known that concussive brain trauma produces a neurometabolic cascade of events (Giza & Hovda, 2001). Furthermore, the metabolic consequences are associated with different time courses which may span several days. Currently, studies are focused on determining those time courses with particular emphasis on identifying inter-injury intervals producing peak vulnerability. Some results have been reported suggesting greater vulnerability when the interval is three days as compared to one day (e.g., Tavazzi et al., 2007; Vagnozzi et al., 2007). While we cannot yet explain the difference in the present studies, we speculate that it may reflect a time course of an effect of the repeated BOP exposures and further study is required to identify the nature of that effect.

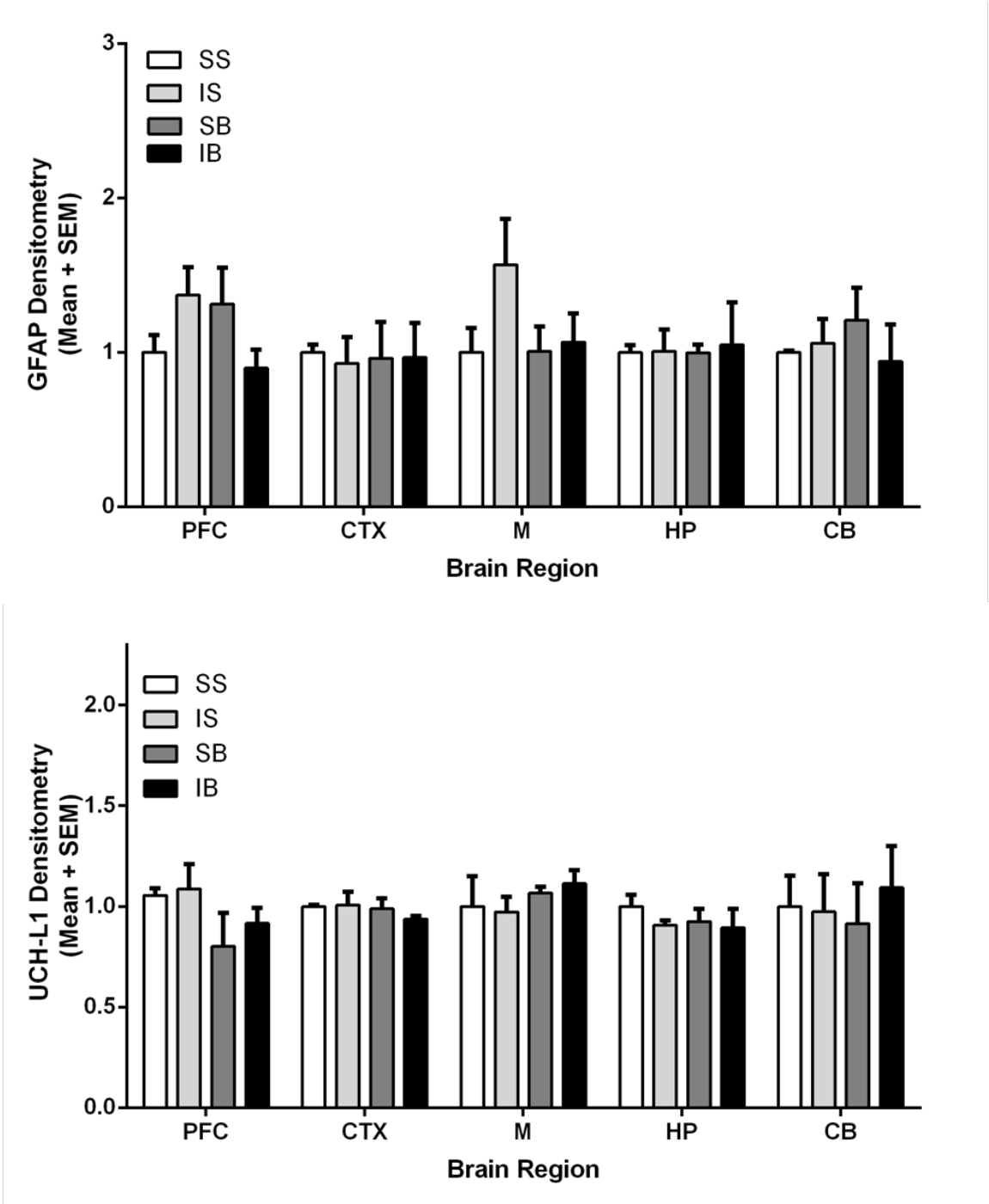

Figure 19. Semi-quantitative Western blotting of GFAP (top) and UCH-L1 (bottom) after fear conditioning (IES) and mTBI (BOP). Densitometry was individually measured in clarified tissue lysates from specific brain regions: prefrontal cortex (PFC), cerebral cortex (CTX), hippocampus (H) and cerebellum (CB). The relative fold change of protein band densitometry quantitation is shown as the average +/- SEM (N = 4-5/group).

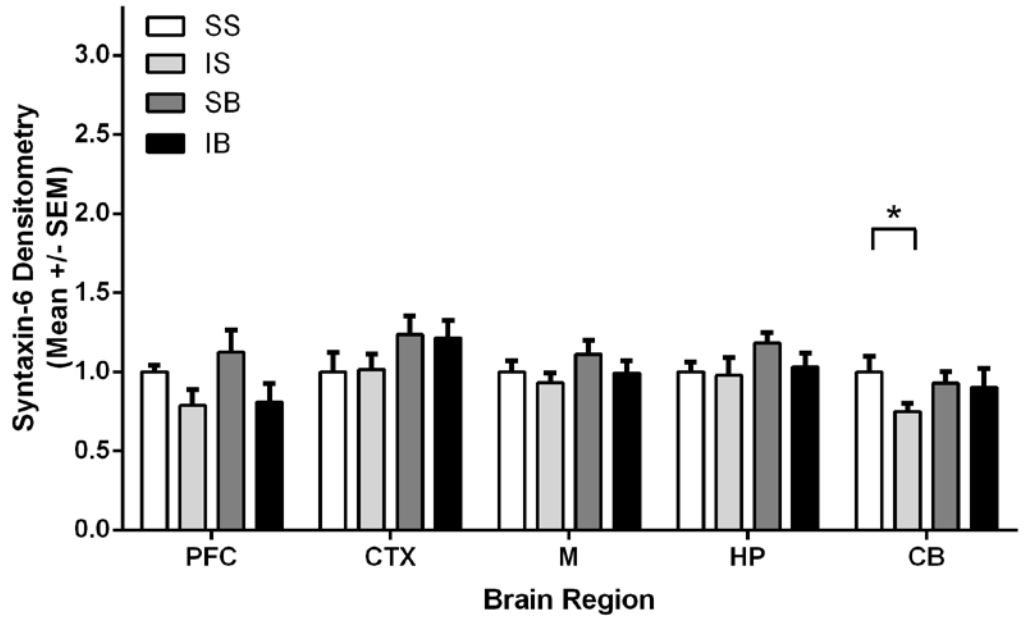

Figure 20. Semi-quantitative Western blotting of Syntaxin-6 after fear conditioning (IES) and mTBI (BOP). Densitometry was individually measured in clarified tissue lysates from specific brain regions: prefrontal cortex (PFC), cerebral cortex (CTX), hippocampus (H) and cerebellum (CB). The relative fold change of protein band densitometry quantitation is shown as the average +/- SEM (N = 4-5/group). Asterisks indicate ($p \leq 0.05$ vs. SS, two-tailed t-test).

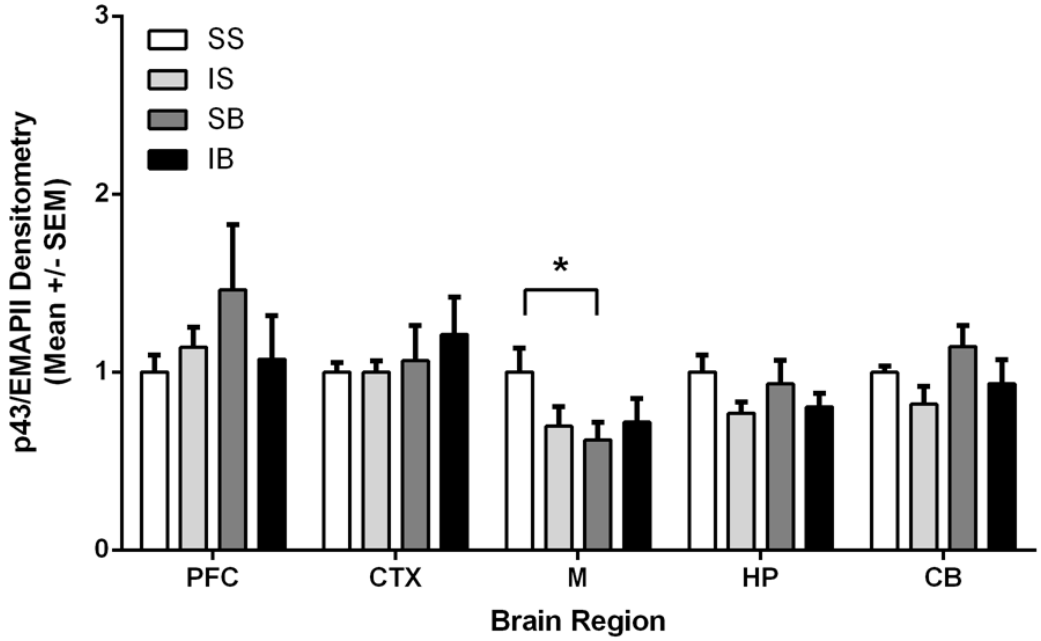

Figure 21. Semi-quantitative Western blotting of p43/EMAP-II after fear conditioning (IES) and mTBI (BOP). Densitometry was individually measured in clarified tissue lysates from specific brain regions: prefrontal cortex (PFC), cerebral cortex (CTX), hippocampus (H) and cerebellum (CB).

The relative fold change of protein band densitometry quantitation is shown as the average +/- SEM (N = 4-5/group). Asterisks indicate ($p \leq 0.05$ vs. SS, two-tailed t-test).

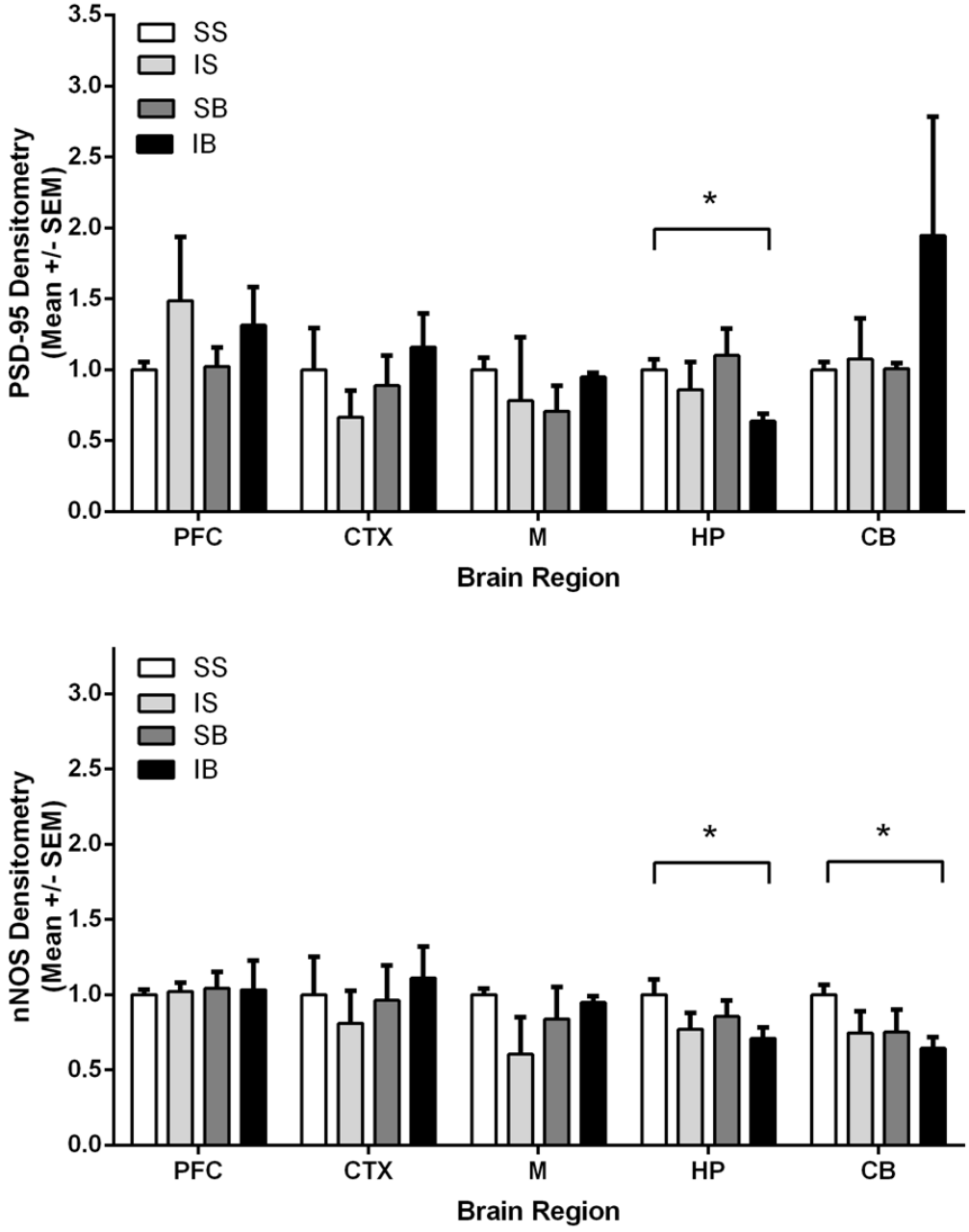

Figure 22. Semi-quantitative Western blotting of PSD-95 (top) and NNOS (bottom) after fear conditioning (IES) and mTBI (BOP). Densitometry was individually measured in clarified tissue lysates from specific brain regions: prefrontal cortex (PFC), cerebral cortex (CTX), hippocampus (H) and cerebellum (CB). The relative fold change of protein band densitometry quantitation is shown as the average +/- SEM (N = 5-6/group). Asterisks indicate ($p \leq 0.05$ vs. SS, two-tailed t-test).

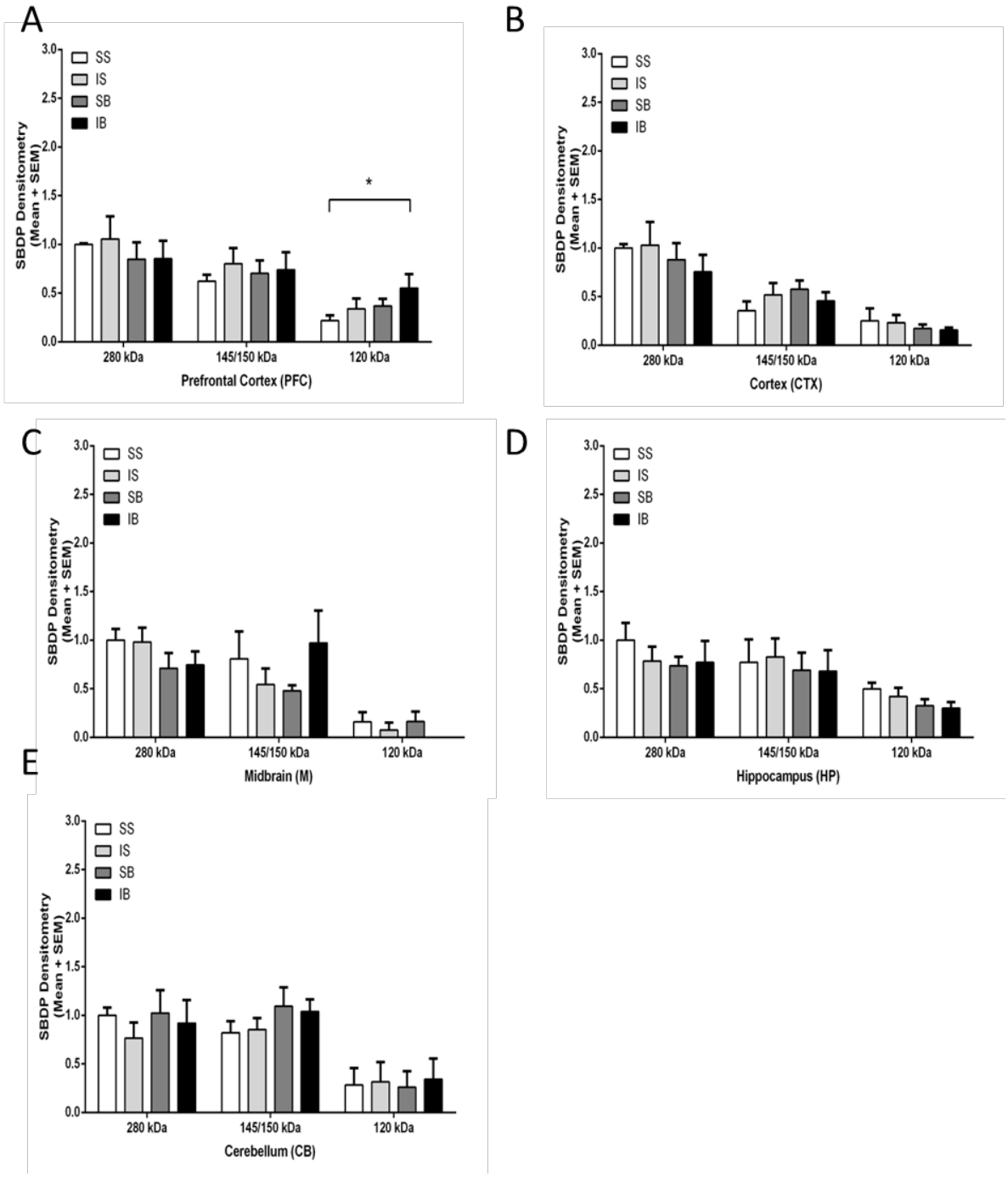

Figure 23. Semi-quantitative Western blotting of α-II Spectrin (280 kDa) and its BDPs (145/150 and 120 kDa) after fear conditioning (IES) and mTBI (BOP). Densitometry was individually measured in clarified tissue lysates from specific brain regions: prefrontal cortex (A, top), cerebral cortex (B, 2nd), midbrain (C, 3rd), hippocampus (D, 4th) and cerebellum (E, bottom/last). The relative fold change of protein band densitometry quantitation is shown as the average +/- SEM (N = 5-6/group). Asterisks indicate ($p \leq 0.05$ One-way ANOVA with Fisher's LSD post-test).

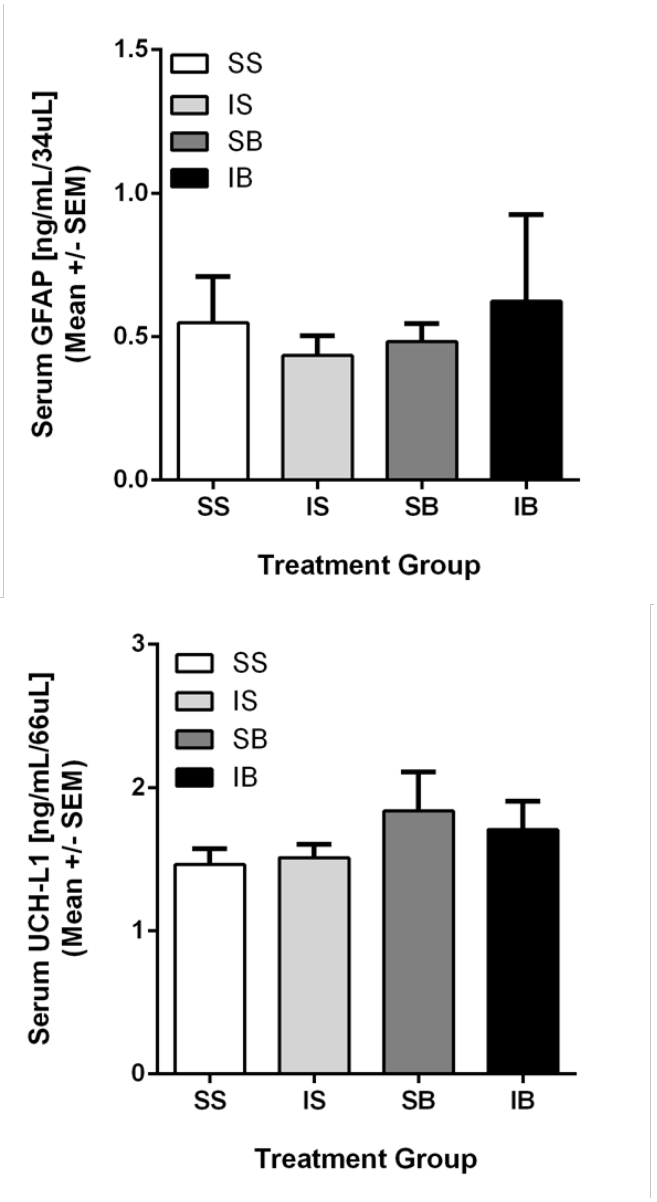

Figure 24. Quantitative ELISA of GFAP and UCH-L1 in serum after predator exposure and/or BOP treatments. Serum samples are from rats 24 h following the last BOP exposure. Data are shown as the mean +/- SEM (N = 5/group).

Proteomics Analysis.

The proteomics studies were initially designed to perform qualitative proteomics of pooled samples from both experiment 1 and experiment 2. However, this would lead to undue duplication of groups treated with SS and SB specifically. More importantly, pooling of samples decreases statistical power. Therefore, this aim of the studies presented focused on conducting proteomics analysis using brain tissue lysates collected from Experiment 1 in order to generate high-quality and quantitative proteomics data using well established protocols that are publishable in a high-ranking,

peer-reviewed scientific journal. The effect of sham IES + Sham BOP (SS), IES + Sham BOP (IS), sham IES + BOP (SB), and IES + BOP (IB) on the cerebral cortex was determined using mass spectrometry based proteomics and spectral counting.

Briefly, proteins were extracted from the cerebral cortex of animals (N = 3) at 24 h after the last BOP event. Proteins were separated by 1-D gel electrophoresis. Excised gel bands representing 10 fractions, parsed by molecular weight, were subjected to in-gel digestion with trypsin followed by reduction and alkylation. Resulting peptides were extracted and analyzed in duplicate by reversed phase nanospray liquid chromatography tandem mass spectrometry (LC-MS/MS). Peptides were searched against a modified forward-reverse concatenated rat database. Thereafter, peptides-protein matches were assembled as count data per protein for each treatment group (SS, IS, SB, and IB) using two search engine – peptide assembly platforms to increase fidelity of identifications and relative count data. The platforms used were (1) Myrimatch with IDPicker (MM-IDP) and (2) Sequest with Scaffold (SQ-SCF). The resulting spectral count data (e.g. the frequency with which any peptide matching a specific protein was detected) was normalized across all replicate samples. Proteins with significant spectral counts (e.g. differences in abundance) were determined by comparing the spectral count value in each treatment group to that of the spectral count value in SS. This strategy was taken to generate a core proteome that consisted of proteins that had high confidence in protein identifications and relative spectral counts.

The number of proteins identified by MM-IDP and SQ-SCF generated from the forward or reverse (decoy) database is indicated (Figure 25, protein IDs, top). MM-IDP identified a much greater number of proteins (3801, 4.02% false discovery rate (FDR)) compared to those generated by SQ-SCF (1311, 0.38% FDR). After exclusion of false positive protein identifications and redundant entries, Venn diagram analysis indicated that 1101 protein identifications were common to both platforms and the experimental treatment groups SS, IS, SB, and SB (Figure 25, core venn diagram, bottom).

This core proteome was then used to define which proteins were increased or decreased as a consequence of IS, SB, or IB treatment at 24 h. Analysis indicated that IS or SB alone led to abundance changes in 50 or 32 proteins, respectfully. IES followed by BOP led to abundance changes of 14 proteins (Figure 26 Venn Treatments). A list of the proteins and their fold change as determined from the two search assembly platforms (MM-IDP and SQ-SCF) after IS, SB, or IB compared to sham is indicated (Tables 5, 6, 7). Protein fold changes ranged from -1.45 to +2.88 in IS, from – 2.44 to +2.89 after SB, and -1.85 to +1.31 based on the two platforms used.

A subset of protein biomarker candidates derived from proteomics analysis were confirmed with multiple reaction mass spectrometry (MRM-MS). For each protein of interest 1-2 tryptic peptides were analyzed. Two peptides each from a control protein, β-III spectrin and target protein (LDH-B) were analyzed. As correctly inferred by discovery based proteomics analysis (Figure 27 open bars), MRM-MS indicates that the fold change of β-III spectrin protein was unchanged as a consequence of treatments (Figure 27 filled bars). Lactate dehydrogenase B (LDH-B) was expected to be decreased after IS, SB and IB (left). MRM-MS confirmed this observation such that LDH-B was, in fact, decreased most strikingly after IS. Further, LDH-B was also less abundant after SB and IB (Figure 27, spectrin and LDH MRM peptides).

Proteins that were significantly increased or decreased were then analyzed by gene set enrichment analysis (GSEA) in the Pathway Studio Program to determine which biological pathways were affected after each treatment (Table 8). After IS, the vast majority of increased proteins were involved in small molecule, protein, carbohydrate turnover. Specifically, small molecule metabolic processes were the most represented (9 proteins increased and 7 proteins increased). Increased proteins also sorted to gene expression (4 proteins) and actin cytoskeleton organization (3 proteins). Proteins that were decreased as a consequence of IS treatment also mapped to small metabolic processes as well as to glycolysis and axon guidance. SB resulted in fewer proteins correlating to specific biological pathways. At maximum, 3 proteins sorted to each of the indicated pathways. Proteins increased in SB also sorted to protein metabolism or proteolysis, axon guidance. Decreased proteins are involved in oxidation-reduction, blood coagulation as well as metabolic and glycolytic processes. Interestingly SB led to down-regulation of mitotic cycle (e.g. G2M transition and cell cycle in general). At most, 3 proteins mapped to each of these pathways as well. Surprisingly, the effect of proteins after IB was not robust. Among increased proteins, only one protein sorted to each of the pathways shown. MHC-II antigen processing/presentation, cell death, platelet activation, axon guidance and blood coagulation were among the biological processes resulting from proteins decreased after IB.

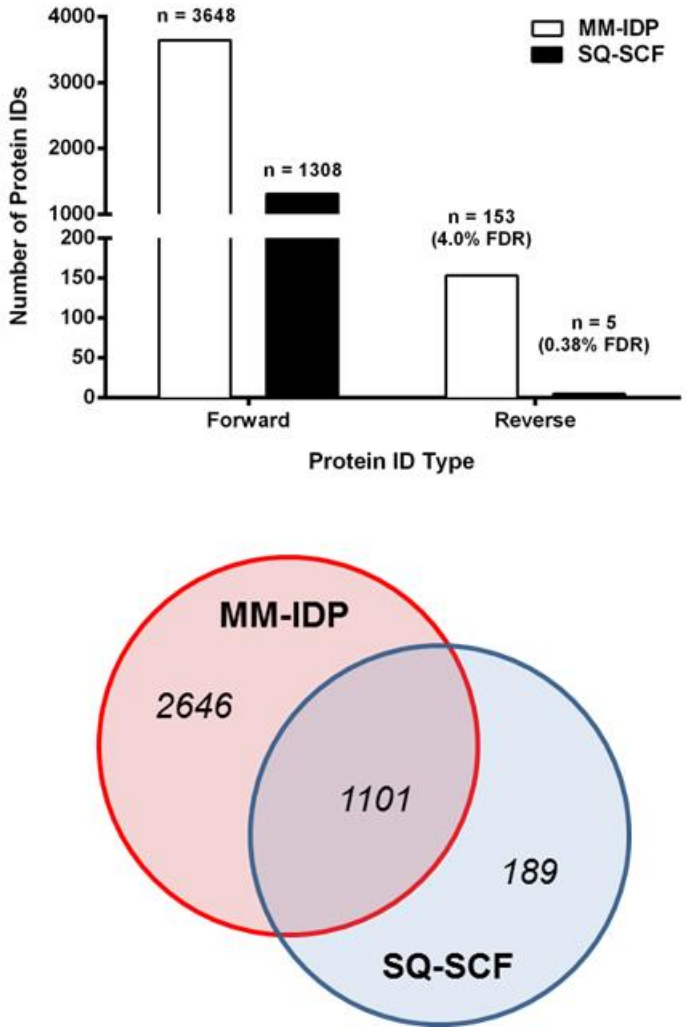

Figure 25. Proteins Detected in the Cerebral Cortex with a Multi-Platform Approach. (Top) The total number of forward and reverse (decoy) proteins identified (n) and false positive rates (% FDR) are displayed for each search-assembly platform. The platform used and treatment group (x-axis) and the number of protein identifications (y-axis) are indicated. (Bottom) Venn diagram of proteins identified by both platforms used for further analysis.

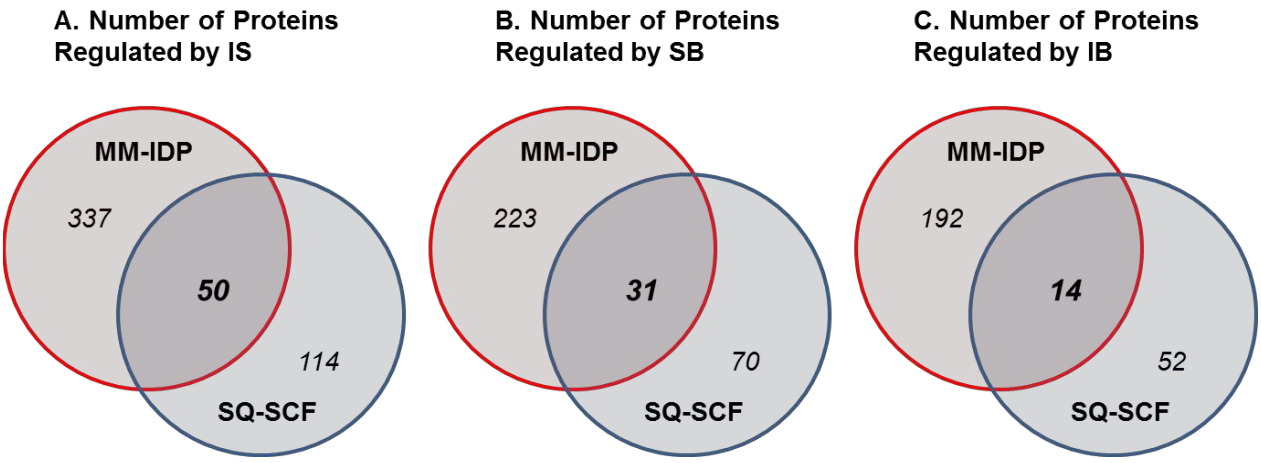

Figure 26. Spectral count fold change distribution of IS, SB, or IB compared to SS for each platform. BioVenn diagrams showing the total number of cerebral cortex proteins with significant changes in abundance based on spectral count compared to Shams. The number of proteins is identified in each injury group: IS (left), SB (center), and IB (right). Proteins were identified and sorted by Myrimatch and IDPicker (MM-IDP, red) or Sequest and Scaffold (SQ-SCF, blue).

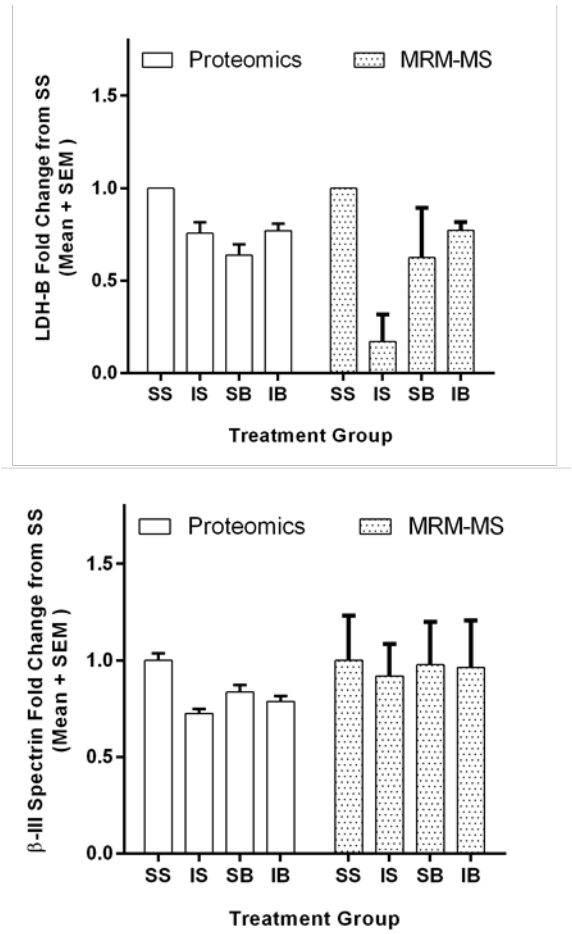

Figure 27. Confirmation of Trends in β-III Spectrin and LDH-B Differential Abundance after IS, SB, or IB. The relative fold change of either β-III Spectrin (top) or LDH-B (bottom) derived from proteomics analysis (left) and MRM-MS peptide quantitation (right).

Table 5 Relative Abundance of Cerebral Cortex Proteins Identified by MM-IDP and SQ-SCF after IS.

			Log $_2$ Fold Change (vs. SS)	
Uniprot Name	Uniprot Entry	Full Protein Name	MM-IDP	SQ-SCF
PRS6B_RAT	Q63570	26S protease regulatory subunit 6B (26S proteasome AAA-ATPase subunit RPT3) (Proteasome 26S subunit ATPase 4) (S6 ATPase) (Tat-binding protein 7) (TBP-7)	-1.45	-0.71
COPB2_RAT	O35142	Coatomer subunit beta' (Beta'-coat protein) (Beta'-COP) (p102)	-1.24	-0.71
D3ZU74_RAT	D3ZU74	Cytoplasmic dynein 1 intermediate chain 2 (Dynein, cytoplasmic, intermediate chain 2, isoform CRA_c)	-1.01	-1.41
D3ZQL7_RAT	D3ZQL7	Protein Tppp (Similar to 25 kDa brain-specific protein (P25-alpha) (Predicted), isoform CRA_a)	-0.57	-0.66
KCRB_RAT	P07335	Creatine kinase B-type (EC 2.7.3.2) (B-CK) (Creatine kinase B chain)	-0.55	-0.58
SPTN1_RAT	P16086	Spectrin alpha chain, non-erythrocytic 1 (Alpha-II spectrin) (Fodrin alpha chain)	-0.48	-0.34
LDHA_RAT	P04642	L-lactate dehydrogenase A chain (LDH-A) (EC 1.1.1.27) (LDH muscle subunit) (LDH-M)	-0.47	-0.50
G3V984_RAT	G3V984	Protein bassoon (RCG25274, isoform CRA_a)	-0.42	-0.48
HXK1_RAT	P05708	Hexokinase-1 (EC 2.7.1.1) (Brain form hexokinase) (Hexokinase type I) (HK I)	-0.42	-0.42
F1MA36_RAT	F1MA36	Spectrin beta 3 (Spectrin beta chain, non-erythrocytic 2)	-0.41	-0.52
MDHC_RAT	O88989	Malate dehydrogenase, cytoplasmic (EC 1.1.1.37) (Cytosolic malate dehydrogenase)	-0.38	-0.51
G3V852_RAT	G3V852	Protein Tln1 (RCG55135, isoform CRA_b)	-0.38	-0.62
G3V8V3_RAT	G3V8V3	Phosphorylase (EC 2.4.1.1)	-0.34	-0.42
G6PI_RAT	Q6P6V0	Glucose-6-phosphate isomerase (GPI) (EC 5.3.1.9) (Autocrine motility factor) (AMF) (Neuroleukin) (NLK) (Phosphoglucose isomerase) (PGI) (Phosphohexose isomerase) (PHI)	-0.30	-0.24
LDHB_RAT	P42123	L-lactate dehydrogenase B chain (LDH-B) (EC 1.1.1.27) (LDH heart subunit) (LDH-H)	-0.29	-0.52
AT1A3_RAT	P06687	Sodium/potassium-transporting ATPase subunit alpha-3 (Na(+)/K(+) ATPase alpha-3 subunit) (EC 3.6.3.9) (Na(+)/K(+) ATPase alpha(III) subunit) (Sodium pump subunit alpha-3)	-0.16	-0.27
AT1A2_RAT	P06686	Sodium/potassium-transporting ATPase subunit alpha-2 (Na(+)/K(+) ATPase alpha-2 subunit) (EC 3.6.3.9) (Na(+)/K(+) ATPase alpha(+) subunit) (Sodium pump subunit alpha-2)	-0.09	-0.27
G3V6V1_RAT	G3V6V1	Aminopeptidase B (Arginyl aminopeptidase (Aminopeptidase B))	0.43	1.10
K6PL_RAT	P30835	ATP-dependent 6-phosphofructokinase, liver type (ATP-PFK) (PFK-L) (EC 2.7.1.11) (6-phosphofructokinase type B) (Phosphofructo-1-kinase isozyme B) (PFK-B) (Phosphohexokinase)	0.49	2.03
IMPCT_RAT	Q5GFD9	Protein IMPACT (Imprinted and ancient gene protein homolog)	0.51	0.88
SCOT1_RAT	B2GV06	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial (EC 2.8.3.5) (3-oxoacid CoA-transferase 1) (Somatic-type succinyl-CoA:3-oxoacid CoA-transferase) (SCOT-s)	0.56	0.56
SFXN1_RAT	Q63965	Sideroflexin-1 (Tricarboxylate carrier protein) (TCC)	0.57	1.13
LANC1_RAT	Q9QX69	LanC-like protein 1 (40 kDa erythrocyte membrane protein) (p40)	0.62	0.62
F1LMP2_RAT	F1LMP2	Merged into Q7TMC7.	0.69	0.59
GNAZ_RAT	P19627	Guanine nucleotide-binding protein G(z) subunit alpha (G(x) alpha chain) (Gz-alpha)	0.74	2.88
PRS8_RAT	P62198	26S protease regulatory subunit 8 (26S proteasome AAA-ATPase subunit RPT6) (Proteasome 26S subunit ATPase 5) (Proteasome subunit p45) (Thyroid hormone receptor-interacting protein 1) (TRIP1) (p45/SUG)	0.75	1.56
G3V8G4_RAT	G3V8G4	Brevican, isoform CRA_a (Protein LOC100910284)	0.76	1.11
CALB2_RAT	P47728	Calretinin (CR)	0.76	2.46
PRS6A_RAT	Q63569	26S protease regulatory subunit 6A (26S proteasome AAA-ATPase subunit RPT5) (Proteasome 26S subunit ATPase 3) (Spermatogenic cell/sperm-associated Tat-binding protein homolog SATA) (Tat-binding protein 1) (TBP-1)	0.78	1.29
G3V940_RAT	G3V940	Coronin	0.91	1.88
B2GV74_RAT	B2GV74	Kinesin light chain 2 (Predicted), isoform CRA_b (Klc2 protein) (Protein Klc2)	0.94	2.10
ARP2_RAT	Q5M7U6	Actin-related protein 2 (Actin-like protein 2)	1.01	1.12
AL7A1_RAT	Q64057	Alpha-aminoadipic semialdehyde dehydrogenase (Alpha-AASA dehydrogenase) (EC 1.2.1.31) (Aldehyde dehydrogenase family 7 member A1) (EC 1.2.1.3) (Antiquitin-1) (Betaine aldehyde dehydrogenase) (EC 1.2.1.8) (Delta1-piperideine-6-carboxylate dehydrogenase) (P6c dehydrogenase)	1.11	1.22
OAT_RAT	P04182	Ornithine aminotransferase, mitochondrial (EC 2.6.1.13) (Ornithine--oxo-acid aminotransferase)	1.21	1.88
COX5B_RAT	P12075	Cytochrome c oxidase subunit 5B, mitochondrial (Cytochrome c oxidase polypeptide Vb) (Cytochrome c oxidase subunit VIA*)	1.22	1.29
VAT1_RAT	Q3MIE4	Synaptic vesicle membrane protein VAT-1 homolog (EC 1.-.-.-) (Mitofusin-binding protein) (Protein MIB)	1.23	1.97
KCY_RAT	Q4KM73	UMP-CMP kinase (EC 2.7.4.14) (Deoxycytidylate kinase) (CK) (dCMP kinase) (Nucleoside-diphosphate kinase) (EC 2.7.4.6) (Uridine monophosphate/cytidine monophosphate kinase) (UMP/CMP kinase) (UMP/CMPK)	1.26	0.57
COX41_RAT	P10888	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial (Cytochrome c oxidase polypeptide IV) (Cytochrome c oxidase subunit IV isoform 1) (COX IV-1)	1.42	1.03
RASN_RAT	Q04970	GTPase NRas (Transforming protein N-Ras)	1.44	1.88
SYRC_RAT	P40329	Arginine--tRNA ligase, cytoplasmic (EC 6.1.1.19) (Arginyl-tRNA synthetase) (ArgRS)	1.68	1.88
TAGL_RAT	P31232	Transgelin (Smooth muscle protein 22-alpha) (SM22-alpha)	1.79	2.75
F1LMT5_RAT	F1LMT5	PEX5-related protein	2.36	2.88
CAN2_RAT	Q07009	Calpain-2 catalytic subunit (EC 3.4.22.53) (Calcium-activated neutral proteinase 2) (CANP 2) (Calpain M-type) (Calpain-2 large subunit) (Millimolar-calpain) (M-calpain)	2.46	2.88

Table 6 Relative Abundance of Cerebral Cortex Proteins Identified by MM-IDP and SQ-SCF after SB.

			Log $_2$ Fold Change (vs. SS)	
Uniprot Name	Uniprot Entry	Full Protein Name	MM-IDP	SQ-SCF
CPLX2_RAT	P84087	Complexin-2 (Complexin II) (CPX II) (Synaphin-1)	-2.44	-1.44
PIPNA_RAT	P16446	Phosphatidylinositol transfer protein alpha isoform (PI-TP-alpha) (PtdIns transfer protein alpha) (PtdInsTP alpha)	-1.77	-1.11
MTPN_RAT	P62775	Myotrophin (Granule cell differentiation protein) (Protein V-1)	-1.56	-1.01
GNAI2_RAT	P04897	Guanine nucleotide-binding protein G(i) subunit alpha-2 (Adenylate cyclase-inhibiting G alpha protein)	-1.09	-1.01
GNAQ_RAT	P82471	Guanine nucleotide-binding protein G(q) subunit alpha (Guanine nucleotide-binding protein alpha-q)	-1.07	-1.41
D3ZU74_RAT	D3ZU74	Cytoplasmic dynein 1 intermediate chain 2 (Dynein, cytoplasmic, intermediate chain 2, isoform CRA_c)	-1.03	-1.49
D3ZQL7_RAT	D3ZQL7	Protein Tppp (Similar to 25 kDa brain-specific protein (P25-alpha) (Predicted), isoform CRA_a)	-1.01	-1.05
GPDA_RAT	O35077	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic (GPD-C) (GPDH-C) (EC 1.1.1.8)	-1.00	-1.04
CAPZB_RAT	Q5XI32	F-actin-capping protein subunit beta (CapZ beta)	-0.91	-1.25
G3P_RAT	P04797	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12) (38 kDa BFA-dependent ADP-ribosylation substrate) (BARS-38) (Peptidyl-cysteine S-nitrosylase GAPDH) (EC 2.6.99.-)	-0.91	-0.51
LDHA_RAT	P04642	L-lactate dehydrogenase A chain (LDH-A) (EC 1.1.1.27) (LDH muscle subunit) (LDH-M)	-0.84	-0.83
STX1B_RAT	P61265	Syntaxin-1B (P35B) (Syntaxin-1B2)	-0.81	-0.71
KCRB_RAT	P07335	Creatine kinase B-type (EC 2.7.3.2) (B-CK) (Creatine kinase B chain)	-0.59	-0.51
LDHB_RAT	P42123	L-lactate dehydrogenase B chain (LDH-B) (EC 1.1.1.27) (LDH heart subunit) (LDH-H)	-0.52	-0.78
DYHC1_RAT	P38650	Cytoplasmic dynein 1 heavy chain 1 (Cytoplasmic dynein heavy chain 1) (Dynein heavy chain, cytosolic) (MAP 1C)	-0.51	-0.49
1433Z_RAT	P63102	14-3-3 protein zeta/delta (Mitochondrial import stimulation factor S1 subunit) (Protein kinase C inhibitor protein 1) (KCIP-1)	-0.49	-0.32
1433E_RAT	P62260	14-3-3 protein epsilon (14-3-3E) (Mitochondrial import stimulation factor L subunit) (MSF L)	-0.49	-0.65
CRYM_RAT	Q9QYU4	Ketimine reductase mu-crystallin (EC 1.5.1.25) (CDK108) (NADP-regulated thyroid-hormone-binding protein)	-0.44	-1.18
G3V984_RAT	G3V984	Protein bassoon (RCG25274, isoform CRA_a)	-0.41	-0.46
F1LRL9_RAT	F1LRL9	Microtubule-associated protein 1B	-0.24	-0.18
HS90B_RAT	P34058	Heat shock protein HSP 90-beta (Heat shock 84 kDa) (HSP 84) (HSP84)	0.41	0.38
G3V881_RAT	G3V881	Leucine rich repeat neuronal 6A, isoform CRA_a (Protein Lingo1)	0.70	1.30
DPP3_RAT	O55096	Dipeptidyl peptidase 3 (EC 3.4.14.4) (Dipeptidyl aminopeptidase III) (Dipeptidyl arylamidase III) (Dipeptidyl peptidase III) (DPP III) (Enkephalinase B)	0.72	1.47
F1LN92_RAT	F1LN92	Protein Afg3l2	0.74	1.25
F1LMP2_RAT	F1LMP2	Merged into Q7TMC7.	0.75	0.61
F1LNN7_RAT	F1LNN7	Merged into G3V8R2.	0.77	0.77
Q1RP74_RAT	Q1RP74	Protein LOC100911774 (RCG53953, isoform CRA_a) (Tubulin folding cofactor B) (ZH14 protein)	0.88	2.06
F1M7B8_RAT	F1M7B8	Protein Ube3a	1.38	3.06
B5DEG8_RAT	B5DEG8	LOC685144 protein (Protein Sec24c) (RCG41932)	1.58	2.69
CAN2_RAT	Q07009	Calpain-2 catalytic subunit (EC 3.4.22.53) (Calcium-activated neutral proteinase 2) (CANP 2) (Calpain M-type) (Calpain-2 large subunit) (Millimolar-calpain) (M-calpain)	2.05	2.89

Table 7 Relative Abundance of Cerebral Cortex Proteins Identified by MM-IDP and SQ-SCF after IB.

			Log $_2$ Fold Change (vs. SS)	
Uniprot Name	**Uniprot Entry**	**Full Protein Name**	**MM-IDP**	**SQ-SCF**
DYHC1_RAT	P38650	Cytoplasmic dynein 1 heavy chain 1 (Cytoplasmic dynein heavy chain 1) (Dynein heavy chain, cytosolic) (MAP 1C)	-0.62	-0.54
F1MA36_RAT	F1MA36	Spectrin beta 3 (Spectrin beta chain, non-erythrocytic 2)	-0.33	-0.36
ENOA_RAT	P04764	Alpha-enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (Enolase 1) (Non-neural enolase) (NNE)	0.24	0.33
HS90B_RAT	P34058	Heat shock protein HSP 90-beta (Heat shock 84 kDa) (HSP 84) (HSP84)	0.56	0.34
D3ZVQ0_RAT	D3ZVQ0	Ubiquitin carboxyl-terminal hydrolase (EC 3.4.19.12)	0.53	0.45
AMPH_RAT	O08838	Amphiphysin	0.47	0.56
G3V852_RAT	G3V852	Protein Tln1 (RCG55135, isoform CRA_b)	-0.69	-1.02
E9PSV5_RAT	E9PSV5	Phosphoserine aminotransferase (EC 2.6.1.52)	-1.31	-1.47
ACTN4_RAT	Q9QXQ0	Alpha-actinin-4 (F-actin cross-linking protein) (Non-muscle alpha-actinin 4)	1.19	1.98
GNAI2_RAT	P04897	Guanine nucleotide-binding protein G(i) subunit alpha-2 (Adenylate cyclase-inhibiting G alpha protein)	-1.10	-1.31
F1LU52_RAT	F1LU52	Protein Dst (Fragment)	-1.85	-1.07
OTUB1_RAT	B2RYG6	Ubiquitin thioesterase OTUB1 (EC 3.4.19.12) (Deubiquitinating enzyme OTUB1) (OTU domain-containing ubiquitin aldehyde-binding protein 1) (Otubain-1) (Ubiquitin-specific-processing protease OTUB1)	-0.70	-0.75
G3V7T3_RAT	G3V7T3	Pantothenate kinase 4	0.57	1.93
D4ABT8_RAT	D4ABT8	Protein Hnrnpul2	1.31	1.03

Table 8 Biological pathways affected after IS, SB or IB.

Directionality	Pathway	# of Entities	Overlap	Overlapping Entities	p-value
Increased	small molecule metabolic process	1278	9	PSMC5;OAT;PFKL;PSMC3;OXCT1;CMPK1;COX5B;BCAN;COX4I1	1.4527E-05
	gene expression	672	4	PSMC5;PSMC3;SRPRB;RARS	0.00946511
	metabolic process	1073	4	PFKL;OXCT1;CAPN2;LANCL1	0.04394425
	actin cytoskeleton organization	185	3	CORO1B;NRAS;ACTR2	0.00159335
	cellular nitrogen compound metabolic process	192	3	PSMC5;OAT;PSMC3	0.00177189
	proteolysis	670	3	PSMC5;CAPN2;RNPEP	0.05132077
	blastocyst development	28	2	PSMC3;CAPN2	0.00056957
	regulation of cellular amino acid metabolic process	51	2	PSMC3;PSMC5	0.00188731
	protein catabolic process	53	2	PSMC3;PSMC5	0.00203663
	negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	65	2	PSMC3;PSMC5	0.00304581
Decreased	small molecule metabolic process	1278	7	MDH1;CKB;GPI;PYGM;PSMC4;LDHA;LDHB	6.6563E-05
	carbohydrate metabolic process	409	5	LDHA;LDHB;MDH1;GPI;PYGM	2.2835E-05
	glycolysis	56	4	GPI;Hk1;LDHB;LDHA	1.5819E-07
	metabolic process	1073	4	ATP1A2;GPI;PYGM;ATP1A3	0.01345083
	cellular carbohydrate metabolic process	10	3	MDH1;LDHB;LDHA	7.1691E-08
	NAD metabolic process	13	3	MDH1;LDHB;LDHA	1.7058E-07
	glucose metabolic process	126	3	GPI;PYGM;MDH1	0.00018235
	axon guidance	331	3	SPTBN2;SPTAN1;TLN1	0.00299456
	oxidation-reduction process	711	3	LDHA;LDHB;MDH1	0.02415189
	transport	1724	3	ATP1A2;DYNC1I2;ATP1A3	0.19700636
Directionality	**Pathway**	**# of Entities**	**Overlap**	**Overlapping Entities**	**p-value**
Increased	cellular protein metabolic process	407	3	SEC24C;TBCB;SRPRB	0.0010549
	proteolysis	670	3	AFG3L2;CAPN2;DPP3	0.00438391
	metabolic process	1073	3	CAPN2;DPP3;ATP2A2	0.01609502
	axon guidance	331	2	NCAN;HSP90AB1	0.01247827
	cell adhesion	633	2	NCAN;ATP2A2	0.04197161
	transport	1724	2	SEC24C;ATP2A2	0.22916985
Decreased	oxidation-reduction process	711	5	YWHAE;GAPDH;LDHA;LDHB;GPD1	0.00054997
	small molecule metabolic process	1278	5	GAPDH;CKB;GPD1;LDHA;LDHB	0.00725661
	transport	1724	5	DYNC1H1;PITPNA;DYNC1I2;STX1B;CPLX2	0.02442323
	carbohydrate metabolic process	409	4	GAPDH;LDHA;LDHB;GPD1	0.000641
	blood coagulation	475	4	GNAQ;CAPZB;GNAI2;YWHAZ	0.00112031
	signal transduction	2736	4	GNAQ;YWHAE;GNAI2;YWHAZ	0.29033848
	glycolysis	56	3	GAPDH;LDHB;LDHA	2.2908E-05
	G2-M transition of mitotic cell cycle	121	3	YWHAE;DYNC1H1;DYNC1I2	0.00022839
	platelet activation	212	3	GNAQ;YWHAZ;GNAI2	0.00117211
	mitotic cell cycle	325	3	YWHAE;DYNC1H1;DYNC1I2	0.00395076
Directionality	**Pathway**	**# of Entities**	**Overlap**	**Overlapping Entities**	**p-value**
Increased	positive regulation of pinocytosis	2	1	ACTN4	0.0006332
	positive regulation of sodium:hydrogen antiporter activity	5	1	ACTN4	0.00158236
	negative regulation of cellular component movement	8	1	ACTN4	0.00253078
	coenzyme A biosynthetic process	8	1	PANK4	0.00253078
	positive regulation of binding	8	1	ENO1	0.00253078
	cellular response to acid	9	1	ENO1	0.00284675
	positive regulation of cell size	9	1	HSP90AB1	0.00284675
	negative regulation of proteasomal ubiquitin-dependent protein catabolic process	10	1	HSP90AB1	0.00316264
	positive regulation of protein import into nucleus, translocation	12	1	HSP90AB1	0.00379416
	protein K48-linked deubiquitination	12	1	USP5	0.00379416
Decreased	antigen processing and presentation of exogenous peptide antigen via MHC class II	97	2	SPTBN2;DYNC1H1	0.00038386
	cell death	172	2	SPTBN2;DYNC1H1	0.00119965
	platelet activation	212	2	GNAI2;TLN1	0.00181423
	axon guidance	331	2	SPTBN2;TLN1	0.00435612
	blood coagulation	475	2	GNAI2;TLN1	0.00879732
	cerebellar Purkinje cell layer morphogenesis	2	1	SPTBN2	0.0006332
	negative regulation of histone H2A K63-linked ubiquitination	3	1	OTUB1	0.00094967
	pyridoxine biosynthetic process	3	1	PSAT1	0.00094967
	L-serine biosynthetic process	5	1	PSAT1	0.00158236
	negative regulation of double-strand break repair	6	1	OTUB1	0.00189858

Discussion and Conclusion, Task 3b.

In Task 3a, rats were first tested for a conditioned fear beginning four days following the last BOP exposure. In study 3b, rats were tested for a conditioned fear only once, and the testing took place on the day following the last BOP exposure. Surprisingly, there was no difference in the expression of conditioned fear between BOP and sham-BOP treatments (i.e., I-B vs. I-S, Figure 18). While a smaller group size was used as compared to task 3a, the difference between the mean values for the suppression indices between the groups in task 3b is very small. It is known that concussive brain trauma produces a neurometabolic cascade of events (Giza & Hovda, 2001). Furthermore, the metabolic consequences are associated with different time courses which may span several days. Currently, studies are focused on determining those time courses with particular emphasis on identifying inter-injury intervals producing peak vulnerability. Some results have been reported suggesting greater vulnerability when the interval is three days as compared to one day (e.g., Tavazzi et al., 2007; Vagnozzi et al., 2007). While we cannot yet explain the difference in the present studies, we speculate that it may reflect a time course of an effect of the repeated BOP exposures and further study is required to identify the nature of that effect.

Targeted biomarkers.

Analysis of tissues and biofluids revealed that well studied biomarkers, GFAP and UCH-L1, were relatively unaffected by treatments. This was not unusual as the treatments were mild in our model of fear conditioning and blast overpressure. The most robust changes are often seen after moderate-severe injuries (Boutte et al., 2012; Diaz-Arrastia et al., 2014; Zoltewicz et al., 2013).

There was an overall loss in several proteins in or near the synaptic densities at 24 h after treatments. Hippocampal PSD-95 loss was evident at 24 h after IB. NNOS loss was more profound and affected in all brain regions and treatment groups. Early (24 h) loss was seen in both the HP and CB after IB. On one hand, postsynaptic densities are clearly affected by IS and IB, but not SB alone particularly after acute (24 h) treatment. In both cases, distal regions of the brain (hippocampus and cerebellum) were most sensitive. Taken together, IS and IB may have short term effects while IS may have long term effects on synapses (Radley et al., 2006). Acute loss of dendrites and axons occurs in mild TBI; (Baalman, Cotton, Rasband, & Rasband, 2013; Merlo et al., 2014) thus, it is clear that even mild BOP in this model leads to degradation. NNOS may serve as a synaptic marker. However, it also plays a role in oxidative-nitrosative stress and is increased after TBI (Bayir et al., 2007; Hall, Wang & Miller, 2012). Loss of nNOS in the

context of IS and IB may also be indicative of synaptic loss in addition to alternations in oxidative stress (Kelley, Balda, Anderson, & Itzhak, 2009).

Proteomics Analysis.

Proteomics analysis offered a platform to discover novel biomarkers of fear conditioning and BOP in an unbiased manner. The method took advantage of two search-assembly platforms to define the effects on core proteome with a high level of confidence in peptide-protein matches. Thereafter, the effect of specific pathways was determined as a consequence of IS, SB or IB and pathway matching. This bioinformatics analysis indicated that IS and SB, compared to IB, had the greatest overall impact on differential protein expression and pathways of the cerebral cortex. Based on proteins that were up and down-regulated after each treatment, an overall alteration in small molecule, carbohydrate and protein metabolism occurs after IS and SB treatment. Both treatments also affected axon guidance. Interestingly, this pathway is decreased after IS, but increased after SB. Further, the actin cytoskeleton organization pathway (primarily referred to as “dendritic”) is positively affected (increased) after IS.

Taken together, this data indicate that both IS and SB injuries affect molecular turnover through slightly different mechanisms. IS may be influenced primarily by this metabolic activity in the mitochondria, which may be a signal of mitochondrial stress, but decreased metabolism involved several cytosolic proteins which may be involved in protein turnover. Overall, changes in amino acid metabolism have been reported in animal models of fear conditioning and TBI (Inoue, Koyama, & Yamashita, 1993; Pascual et al., 2007). Furthermore, metabolism of glucose is altered in TBI patients (Luyten et al., 2012; Stocker et al., 2014). Closer inspection the metabolism pathway in SB indicates that proteins in this group are involved in transport and secretion of nascent proteins. Pathways analysis infers that both axons and dendrites are affected by IS, but only axons are affected by SB. IB did not extraordinarily exacerbate or mitigate the pathways influences by either IS or SB, indicating that, from the perspective of inducing an injury, IB is quite unique from IS or SB alone.

KEY RESEARCH ACCOMPLISHMENTS:

- Evaluated the effects of mTBI from blast overpressure, and traumatic stress from predator exposure, alone and in combination, on indices of cognition and exploration in rats.

- Characterized the effects of mTBI from blast overpressure and traumatic stress from predator exposure, alone and in combination, on food-maintained operant performance and on the expression and extinction of a conditioned fear.

- Completed targeted spatial-temporal analysis of protein biomarkers after either fear conditioning or predator stressors and mild blast over pressure.

- Determined the effect on the cerebral cortex proteins and pathways after fear conditioning and mild injury using unbiased, proteomics based discovery.

CONCLUSION:

The set of studies executed in this project used an mTBI from a specific blast over pressure (75 kPa X 3). One general and consistent conclusion from the studies is that this regimen is, indeed, a mild insult and is probably at, or near, the lowest level of insult that will produce any behavioral effects, based upon the results of several different performance measures at several different time points following presentation.

Within the qualifications of the mTBI intensity above, no evidence was found that a predator stressor had a synergistic effect with the mTBI.

Predator stressors or fear conditioning and blast over pressure (either alone or in tandem) lead to a myriad of differential abundance changes of brain tissue protein biomarkers. Predator stress alone may be correlated to pTau levels in blood. Brain proteins were uniquely, not synergistically, affected by either stress or blast overpressure treatments. Although treatments converged upon several common pathways, the specific proteins involved in each treatment were quite unique.

A particularly interesting and significant finding from the project is that mTBI decreased inhibitory control in a conditioned fear procedure. Recently, this disinhibition from mTBI was also observed in another rodent species using different procedures (Ojo et al., 2014). That is, there was a convergence of preclinical data in this regard. The disinhibitory effects of mTBI may be a very sensitive behavioral measure and merits further examination in a preclinical and clinical context.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

(1) Lay Press:
(2) Peer-Reviewed Scientific Journals:
(3) Invited Articles:
(4) Abstracts:

(2) R.F. Genovese, L.P. Simmons, S.T. Ahlers, E. Maudlin-Jeronimo, J.R. Dave, A.M. Boutte, Effects of Mild TBI from Repeated Blast Overpressure on the Expression and Extinction of Conditioned Fear in Rats, Neuroscience (2013), doi: http://dx.doi.org/10.1016/j.neuroscience.2013.09.021

(2) A.M. Boutté, S.F. Grant and J.R. Dave, A., Simplified Workflow for Protein Quantitation of Rat Brain Tissues using Label-free Proteomics and Spectral Counting. Injury Models of Central Nervous system: Traumatic Brain Injury, Methods in Molecular Biology, (Humana Press, Inc.) (in review, Nov 2014).

(4) A.M. Boutte´, M. Tong, R. Pedersen, M. Shaughness, E. Maudlin-Jeronimo, K. Schmid, R.F. Genovese, S.T. Ahlers, F.C. Tortella and J.R. Dave, Differential Protein Changes in Penetrating and Non-penetrating Models of TBI. National Neurotrauma Society meeting, Phoenix, AZ 2012.

(4) A.M. Boutté, J. Guingab-Cagmat , E. Mauldin-Geronimo, L.P. Simmons, S.T. Ahlers, R.F. Genovese, F.C. Tortella, K.E. Schmid, J.R. Dave, Multi-platform Data Integration of the Cerebral Cortex Proteome in Rodent Models of Fear Conditioning and Repetitive Blast. American Society for Mass Spectrometry and Allied Topics Annual Conference, 2014.

(4) A.M. Boutté, J. Guingab-Cagmat, E. Maudlin-Jeronimo, Y. Chen, L.P. Simmons, S. T. Ahlers, R.F. Genovese, F.C. Tortella, K.E. Schmid and J.R. Dave, The Cerebral Cortex Proteome in Rodent Models of Fear Conditioning and Repetitive Blast. Society for Neuroscience Annual Meeting, 2014.

List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

R. F. Genovese, A. M. Boutté, Neurocognitive and Biomarker Evaluation of Combination mTBI from Blast Overpressure and Traumatic Stress, TBI Biomarkers IPR, 1 Apr 2014, Ft. Detrick, MD.

INVENTIONS, PATENTS AND LICENSES:

Nothing to report.

REPORTABLE OUTCOMES:

Nothing to report.

OTHER ACHIEVEMENTS:

Nothing to report.

APPENDICES:

Effects of Mild TBI from Repeated Blast Overpressure on the Expression and Extinction of Conditioned Fear in Rats, Neuroscience (2013)

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APPENDICES:

A1. Acronym and Abbreviation Definitions

BOP: Blast overpressure. In our procedure, we are using three exposures at 75 kPa (~10.8 psi).

CER: Conditioned emotional response. With regard to the conditioned fear procedure, it refers to the conditioned response (CR).

CR: Conditioned response. The response elicited by the CS alone following pairing with a US. In the conditioned fear procedure, the conditioned response is assumed to include “fear.”

CS: Conditioned Stimulus. With regard to the conditioned fear procedure, it refers to the flashing lights and pulsing tone stimuli that are paired with IES initially and subsequently presented alone in the VI32.

CSF: Cerebral spinal fluid.

CTX: Cerebral cortex.

GFAP: Glial fibrillary acidic protein.

HP: Hippocampus.

I-B: IES + BOP. The treatment condition where rats receive the CS paired with IES (the US) and BOP.

I-S: IES + sham BOP. The treatment condition where rats receive the CS paired with IES (the US) and sham BOP.

IES: Inescapable electric shock. In our procedure, the CS is paired with the IES, which constitutes the US, to produce the fear conditioning.

mTBI: Mild traumatic brain injury. In our project the mTBI is produced by the BOP.

PFC: Prefrontal cortex.

SBDP: Spectrin break-down product.

S-B: Sham IES + BOP. The treatment condition where rats receive the CS only (no IES / US) and BOP.

S-S: Sham IES + sham BOP. The treatment condition where rats receive the CS only (no IES / US) and sham BOP.

SI: Suppression index. A measure to evaluate the degree of response suppression on the conditioned fear procedure, i.e., a measure of the magnitude of the CER. Calculated by the formula: (response rate before - response rate after) / (response rate before + response rate after). A suppression index is usually calculated for 1- and 3- min intervals before and after presentation of the CS.

UCH-L1: Ubiquitin carboxy-terminal hydrolase-L1.

VI32: Variable-interval 32 second schedule of reinforcement. The operant conditioning schedule specifying that one lever press following an average interval of 32 sec produces reinforcement. Individual intervals are normally distributed around a mean of 32 seconds.

A2: Methods and Procedures

All studies were conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adhere to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 and 2013 editions. All procedures were reviewed and approved by the Institutes' Animal Care and Use Committees, and performed in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

1.0 Animals.

Adult male Sprague-Dawley rats or male hooded rats (MWM study only) (Charles River, Wilmington, MA) were used. Rats were individually housed in a temperature-controlled environment under a 12L: 12D cycle (lights on at 06:00 h) and water was always available in the home cages.

For the studies using food restriction, body weights were maintained at approximately 325 g by food administered during experimental sessions and supplemental feedings (PMI Nutrition International, St. Louis, MO) occurring several hours after experimental sessions. All rats were weighed daily, Monday-Friday.

2.0 Behavioral procedures.

2.1 Morris Water Maze.

Further details of the Morris water maze procedure may be found in earlier reports from the partnering PI. Briefly, rats learn to navigate an "open field" pool to reach a platform to exit the water. Sessions are conducted as four blocks of four trials with the latency to find the platform as the major dependent measure. The ability of the rats to learn the maze, as evidenced by a significant decrease in latency with successive trials is evaluated. Additionally, the acquisition functions are compared between treatments to determine a possible effect of the treatments.

2.2 Predator exposure.

The procedure involved exposing rats, in a protected fashion, to three different predators (snake, ferret and cat) sequentially. For each exposure, the rat is in close proximity to, but not in direct contact with, the predator such that the possibility of physical injury is prevented. Rats were exposed once to each predator and the order of predators is snake (15 min), ferret (5 min), and cat (15 min).

For all predator exposures, rats are placed in covered transfer cages and brought from the home cage to the room housing the predator. Following the exposure, they are transferred back to their home cage. At least one Investigator is present for the duration of each exposure.

Snake (Boa constrictor) Exposure: For the snake exposure procedure, rats are first placed into a protective, clear acrylic, cylindrical rodent restrainer (Harvard Apparatus, Holliston, MA, or equivalent) designed for rodents weighing up to 700g and measuring 3-1/4" H and 7-9/10" L. The functional length of the restrainer can be reduced to 5-1/4" by repositioning the head gate and is positioned for each rat to allow the least amount of restraint. The restrainer is mounted on a flat base providing stability and making the container resistant to displacement by the snake. The restrainer also has a series of holes on each side as well as on the head and tail gates. The rat is inserted into the holder along with some material from the snake's home cage (e.g., shed snake skin or several chunks of bark from the snake's home cage). An adjustable strap is then wrapped around the length of the restrainer to prevent the possibility that the rat could escape from the holder.

After the rat is placed in the aforementioned protective restrainer, it is placed inside the container normally used for feeding the snake. That is, normal practice for feeding the snake is to move the snake from the home cage to a large plastic container (Sterilite™ , 85.7 cm x 49.2 cm x 34.0 cm) specifically for feeding (deceased rats obtained commercially). The rat is placed into the feeding container with the snake for 15 min. Subsequently, the rat is removed from the feeding container and returned to the transfer cage and then back to the home cage.

Ferret (Mustela putorius furo) Exposures: Rats are exposed to three ferrets (group housed) in the home cage of the ferrets. For the exposure, a rat is first placed into a protective exposure tube measuring 63.5 cm in length. Except for structural supports that also prevent the tube from rolling, the tube consists of a wire mesh having an inside diameter of 13.5 cm. The mesh that forms the walls of the tube has a grid of 0.64 cm x 0.64 cm openings. These holes are large enough to allow visual, auditory and olfactory cues to reach the rat inside, but not so large as to allow injurious physical contact by the ferrets.

All inside accessories (e.g., ferret hammocks and toys) are first removed from the ferret cage and the rat is secured inside the protective restrainer. The protective container, with the rat inside, is then placed in the center of the home cage of the ferrets for 5 min. Subsequently, the rat is removed from the ferret cage and returned to the transfer cage and then back to the home cage.

Cat (Felis cattus) Exposure: Rats are exposed to cats in the home cage of the cats. Four cats were used and were pair housed. Exposures were conducted in the two home cages of the cat pairs, and were balanced such that both pairs of cats were used within each treatment group as an experimental design guideline.

For the exposure, a rat is placed into a protective exposure tube measuring 122.0 cm in length. Except for structural supports that do not allow the tube to roll, the tube consists of a wire mesh having an inside diameter of 14.0 cm. The mesh that forms the walls of the tube has a grid of 2.5 cm x 2.5 cm openings. These holes are large enough to allow

visual, auditory and olfactory cues to reach the rat inside, but not so large as to allow injurious physical contact by the cats. The tube was placed on a stainless steel pan which exceeds the length and width of the protective exposure tube. A small amount of used cat litter is placed along the middle of the stainless steel pan. The rat is then placed into the protective exposure tube and the tube is placed on the stainless steel pan and then both are brought into the home cage of the cats for 15 min. During the exposure, small quantities of dry cat food or food treats are placed on the outside perimeter of the stainless steel pan (away from the used litter) to insure that the cats maximize their proximity to the rat. After the exposure, the rat is removed from the home cage of the cats and returned to the transfer cage and then back to the rat's home cage.

Sham exposure: Sham or control exposures are conducted using duplicate exposure containers precisely as previously described. A sham treatment will, therefore, consist of three exposure sessions using exposure containers identical in dimension and structure as those used for snake, ferret and cat exposures but that are used specifically for the sham treatments and are separate from those used for the predator exposures. Each sham exposure was for the same duration as the respective predator exposure. Sham exposures took place in a room that has never housed a predator and was typically in an adjacent room that is normally used to house the rats. The rat was placed in the sham holder and then placed in a quiet area of the room. After the exposure, the rat is returned to the home cage.

2.3 Elevated plus maze.

The elevated plus maze (EPM) procedure uses a commercial (Kinder Scientific, Poway, CA) four arm maze where the arms are arranged in a "plus sign" configuration. Each arm measures 56.0 cm L x 11.5 cm W. Two of the arms, which face each other, are "closed". That is, these arms have side walls measuring 45.7 cm high and no ceiling. The remaining two arms, which also face each other, are "open". That is, they do not have side walls or a ceiling, only a floor. The legs of the four arms elevate the maze to a height of 79.0 cm from the floor. Associated electronic interface equipment is contained under the center of the maze. The arms, floor and walls of the maze are constructed of tinted plastic and the legs are constructed of stainless steel. There is a grid of infra-red photo emitters and detectors under the surface of the floor of all arms of the maze. Along with associated electronics, this grid is used to track location and movement of the rat in the maze.

The EPM procedure was run with the overhead lights turned off and only dim lighting provided by a small bench lamp. A session began with the rat placed in the intersection of the four arms (i.e., the center of the maze). The rat is then left undisturbed and allowed to freely explore the maze for the entire session which was 5 min in duration.

Sessions were controlled and monitored by a dedicated instrumented controller which also collects and saves data files for each session. The major dependent measure on the test is movement counts. Additional dependent measures include movement and time in closed arms, open arms and intersection portions of the maze.

2.4 Variable Interval Schedule of Reinforcement.

Sessions for the variable-interval 32 second schedule of reinforcement (VI32) were conducted in ten standard rodent operant conditioning chambers (model ENV-008 or equivalent, Med Associates, St. Albans, VT) housed in ventilated, light- and sound-attenuating cubicles. Each chamber contained two response levers and a food trough attached to a food dispenser capable of delivering 45 mg food pellets (F0021, Bio-Serv, Frenchtown, NJ). Each chamber also contained a house light, mounted on the front wall near the ceiling, a stimulus light mounted above each of the response levers and a Sonalert® tone generator (2.8 kHz, model ENV-223 AM or equivalent, Med Associates, St. Albans, VT). Experimental events were controlled and monitored by a microcomputer, using Med-PC® control software (Med Associates, St. Albans, VT).

Rats were initially trained to lever-press for food pellets under a continuous schedule of reinforcement. Although two levers were present in each chamber, only one lever produced food reinforcement. In this regard, an equal number of boxes were designated with the active lever on the left and on the right. When lever pressing was maintained by food presentation, rats were trained to lever-press under a VI32 schedule of food reinforcement. The schedule specifies that a single lever-press, following an average interval of 32 sec, produces food reinforcement (i.e., a single food pellet). Interval values for the schedule were chosen pseudo-randomly, without replacement, from a set of values that followed a normal distribution (range=0.8-127.9sec). Normal VI32 sessions were always conducted without illuminating the house light or the stimulus lights above both levers. All sessions were 30 min in duration.
When responding under the VI32 was stable (as judged by inspection of the daily response rates and cumulative response records), rats were assigned to a treatment group. In all cases, at least 60 training sessions were conducted before assignment. Assignment was balanced with respect to rate of responding with the objective that each treatment group would have similar average rates of responding on the task.

2.5 Conditioned Suppression / Conditioned Fear.

Conditioned fear training took place in a stainless steel chamber measuring 109 cm x 66 cm x 97 cm placed inside a ventilated, sound- and light-attenuating cabinet. The chamber contained a grid floor consisting of stainless steel rods running along the width and electric shock stimuli were presented through these rods. Electric shock stimuli were generated by a Programmable Shocker (Lafayette Instrument Company, IN, model HSMSCK). The device was used to output an isolated and scrambled (4-pole), constant current electrical stimulus calibrated to 1.0 mA. The onset and duration of the shock stimulus was controlled by a laptop computer and associated interface equipment using custom software. The chamber also contained two house lights, four stimulus lights and a Sonalert ® tone generator, identical to those in the chambers used for the VI32 sessions.

Rats were placed inside the chamber for a 35 min session in which 20 electric shock stimuli were presented at random times during the session with the exclusion of the first and last 2.5 min of the session and with at least 30 sec between shocks. Each shock stimulus was 1.0 mA in intensity and 2.0 sec in duration. A conditioned stimulus (CS) was presented 0.5 sec prior to the onset of the shock stimulus, and continued for the duration of the shock stimulus (i.e., 2.5 sec total). The CS consisted of intermittently operating the Sonalert (0.35 sec on, 0.15 sec off), house light (0.25 sec on, 0.25 sec off) and stimulus lights (0.15 sec on, 0.35 sec off). For sham controls, sessions were conducted without the shock stimuli but with all other stimuli and parameters.

2.6 VI32 + CS Procedure (Extinction / CER testing).

Sessions for the CR testing procedure were conducted in an identical fashion as the VI32 sessions described previously except that a CS (flashing lights and a pulsing tone) lasting 2.5 sec was presented once during the session. The CS presented was essentially identical to that used during the inescapable electric shock procedure. That is, the CS consisted of intermittently operating the Sonalert (0.35 sec on, 0.15 sec off), house light (0.25 sec on, 0.25 sec off) and stimulus lights (0.15 sec on, 0.35 sec off). The CS was presented at a time randomly chosen from an array of times following a normal distribution but excluding the first and last 6 minutes of the 30 min session.

3.0 Blast overpressure exposure.

Rats were exposed to overpressure using a shock tube and associated air blast system. The shock tube has a 12-inch circular diameter and is a 19.5 ft long steel tube divided into a 2.5 ft compression chamber that is separated from a 17 ft expansion chamber. The compression and expansion chambers are separated by polyethylene Mylar TM sheets that control the peak pressure generated. The peak pressure at the end of the expansion chamber was determined by piezoresistive gauges specifically designed for pressure-time (impulse) measurements (Model 102M152, PCB, Piezotronics, Inc., Depew, NY, USA).

Rats were first anesthetized using an isoflurane gas anesthesia system consisting of a vaporizer, gas lines and valves, and an activated charcoal scavenging system. For the anesthesia, rats were placed into a polycarbonate induction chamber, which was closed and immediately flushed with a 5% isoflurane mixture in air for two minutes. Rats were then placed into a cone-shaped plastic restraint device and then placed into the shock tube. Movement was further restricted during the blast exposure using 1.5 cm diameter flattened rubber tourniquet tubing as restraint straps. Three such straps were spaced evenly to secure the head region and upper and lower torso, while the animal was in the plastic restraint cone. The end of each strap was threaded through a toggle and run outside of the exposure cage where it was tied to prevent movement during the blast overpressure exposure without restricting breathing. Rats were positioned with the head facing the blast exposure without body shielding to produce a full body exposure to the blast wave. Blast exposed animals received 74.5 kilopascal (kPa) exposures equivalent to 10.8 pounds per square inch (psi). One exposure per day was administered for three

consecutive days. Sham exposed animals were treated identically except that they did not receive a blast exposure.

4.0 Tissue Harvesting.

4.1 Euthanasia.

A single dose mixture containing 70 mg/kg ketamine and 6 mg/kg xylazine was administered to the rat via intramuscular injection using a 24-26 gauge needle. The dose generally induces deep anesthesia within 5 min and lasts for about 60 min, making it the preferred anesthetic agent for trans-cardial blood and cerebral spinal fluid (CSF) collection. Before making any incisions, an adequate level of anesthesia was verified by checking for loss of consciousness and failure to react to a noxious stimulus, such as a pinch on the tail with a pair of forceps. In all rats, blood and CSF samples are taken for biomarker analysis. Additionally, after euthanasia, brain tissue from each rat is collected for potential proteomic analysis.

4.2 Biosample collection.

For CSF collection, a 4-cm midline incision is made from 0.5 cm anterior to the interauricular line. The atlanto-occipital dura mater is exposed by separating the nuchal muscles, and CSF is collected by a 30 G syringe needle through the skull. Blood is collected by cardiac puncture. Both CSF and blood samples are collected into heparin coated tubes in the presence of protease/phosphatase inhibitors and stored on ice. A separate cohort of blood is collected in serum clotting tubes for 30 minutes. Serum is transferred to clean tubes and supplemented with protease/phosphatase inhibitors. All biofluids are centrifuged at 1200 g for 10 min at 4°C. The resulting plasma, serum, or clarified - cell free CSF is transferred to Eppendorf tubes and stored at -80° C. Select brain regions (prefrontal cortex, cerebral cortex, midbrain hippocampus, and cerebellum) of both left and right hemispheres are dissected, flash frozen in N2 (l), and individually stored at -80° C until processing.

4.3 Western Blotting of Brain Tissues.

Brain tissue regions from the left hemisphere are sonicated for 2 X 10 s in 1 X RIPA lysis buffer containing protease and phosphotase inhibitors (Sigma, St. Louis, MO) and centrifuged at 10 kg, 4° C, 10 minutes. Clarified supernatant is collected and protein concentrations are determined by using the BCA protein assay kit (Thermo/Pierce, Rockford, IL). Samples containing 10 µg of protein are denatured, reduced with dithiothreitol (DTT), loaded and separated by 4–15% gradient polyacrylamide gel electrophoresis (PAGE) with the NuPage system (Invitrogen, Grand Island, NY). After transferring to PVDF membranes, blots are probed with primary antibodies to each protein biomarker. Densitometry of protein band intensity is measured using an ImageQuant LAS 4000 with automated background subtraction (GE Healthcare, Piscataway, NJ).

4.4 Enzyme Linked Immunosorbent Assays.

Protein samples extracted from brain tissues are prepared as with Western blotting, but normalized to contain 10 µg of total protein and 0.25 X RIPA per well. Tissue GFAP or UCH-L1 is determined using commercially available kits containing internal standards as described by the manufacturer (USCNK/Life Science; Cedarlane Laboratories, Burlington, NC). All biological samples are measured in duplicate using a colorimetric plate reader (450nm).

4.5 Biofluid Preparation.

Equal volumes of CSF (60uL) or serum (35uL) are diluted 10-fold and albumin and immunoglobulins are depleted using ProteoExtract resins (Millipore). Eluted protein solutions are concentrated with 3 kDa MWCO spin filters (Millipore) and the final volumes normalized. Total protein content in serum is estimated with the BCA kit (Thermo/Pierce). Serum is further normalized by volume and protein concentration prior to analysis. All samples are stored at -80°C until testing. These samples are tested for biomarkers using custom ELISA designed in-house.

4.6 Proteomics Analysis of Brain Tissues.

To optimize protein detection and differential protein analysis, we have modified the proteomics protocol slightly from our original plan. The right hemisphere of the cerebral cortex is isolated as in section 4.3. Samples are prepared as stated for Western blotting and 20ug is loaded per sample per lane. After reducing and denaturing PAGE, gels are stained with Colloidal Blue dye (Invitrogen, Carlsbad, CA). Protein bands are excised and parsed by molecular weight ranges. Each gel-piece containing protein is reduced, alkylated, and then digested with trypsin. The resulting peptides are extracted with acidified 50% acetonitrile, dried, and stored at -80 °C until use. Lyophilized peptides are re-constituted and then analyzed by shotgun proteomics. After database searching, differential protein abundance due to treatments is determined by spectral counting. Gene set enrichment analysis (GSEA) is then performed using Pathway Studio Web (v10.5.0.5) on the resulting peptide-protein matches to determine which overall pathways are affected by each treatment. Proteins that are significantly increased or decreased will be confirmed with mass spectrometry or immune-based methods such as Western blotting or ELISA.

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EFFECTS OF MILD TBI FROM REPEATED BLAST OVERPRESSURE ON THE EXPRESSION AND EXTINCTION OF CONDITIONED FEAR IN RATS ☆

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Abstract—Mild traumatic brain injury (mTBI) and post-traumatic stress disorder (PTSD) are pressing medical issues for the Warfighter. Symptoms of mTBI can overlap with those of PTSD, suggesting the possibility of a causal or mediating role of mTBI in PTSD. To address whether mTBI can exacerbate the neurobiological processes associated with traumatic stress, we evaluated the impact of mTBI from a blast overpressure (BOP) on the expression of a conditioned fear. In the rat, conditioned fear models are used to evaluate the emotional conditioning processes that are known to become dysfunctional in PTSD. Rats were first trained on a variable interval (VI), food maintained, operant conditioning task that established a general measure of performance. Inescapable electric shock (IES) was paired with an audio-visual conditioned stimulus (CS) and followed 1 day later by three daily exposures to BOP (75 kPa). Subsequently, the CS alone was presented once every 7 days for 2 months, beginning 4 days following the last BOP. The CS was presented during the VI sessions allowing a concurrent measure of performance. Treatment groups (n = 10, each group) received IES + BOP, IES + sham-BOP, sham-IES + BOP or sham-IES + sham-BOP. As expected, pairing the CS with IES produced a robust conditioned fear that was quantified by a suppression of responding on the VI. BOP significantly decreased the expression of the conditioned fear. No systematic short- or long-term performance deficits were observed on the VI from BOP. These results show that mTBI from BOP can affect the expression of a conditioned fear and suggests that BOP caused a decrease in inhibitory behavioral control. Continued presentation of the CS produced progressively less response suppression in both fear conditioned treatments, consistent with extinction of the conditioned fear. Taken together, these results show that mTBI from BOP can affect the expression of a conditioned fear but not necessarily in a manner that increases the conditioned fear or extends the extinction process. Published by Elsevier Ltd. on behalf of IBRO.

Key words: mTBI, blast overpressure, conditioned fear, PTSD, stress processes, operant conditioning.

INTRODUCTION

Post-traumatic stress disorder (PTSD) and mild traumatic brain injury (mTBI) are significant health concerns for the Warfighter. A substantial percentage of individuals exposed to mTBI experience persistent symptoms (i.e., post-concussive syndrome) and both TBI and mTBI have been associated with psychiatric disorders including major depressive disorder (Vanderploeg et al., 2007; Bombardier et al., 2010). It has also been observed that mTBI is associated with the subsequent occurrence of PTSD (e.g., Hoge et al., 2008). The occurrence of mTBI on the battlefield, however, is also typically associated with psychological trauma which further complicates the delineation. Nevertheless, the association and overlap in symptoms between mTBI and PTSD has raised the possibility that mTBI could mediate or, in some manner, predispose an individual to PTSD. While the neurobiology of such a relationship has not been demonstrated, some mechanisms have been proposed (Simmons and Matthews, 2012).

There are, however, only a few preclinical laboratory studies investigating the relationship between mTBI and animal models of PTSD. For example, Reger et al. (2012) evaluated conditioned fear in rats several days after a fluid percussion injury. Using several conditioned freezing procedures, they reported an increased conditioned fear response (i.e., an increased freezing time) due to injury. Elder et al. (2012) found behavioral

☆ Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official, or as reflecting true views of the Department of the Army, the Department of the Navy or the Department of Defense. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 and 2011 editions.

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Abbreviations: ANOVA, analysis of variance; BOP, blast overpressure; CR, conditioned response; CS, conditioned stimulus; IES, inescapable electric shock; mTBI, mild traumatic brain injury; PTSD, post-traumatic stress disorder; VI, variable interval (schedule of reinforcement).

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changes in rats 6 weeks after mTBI from blast overpressure (BOP) which they interpret as an increase in ''PTSD-like traits''. The behavioral changes included decreased exploratory activity on an elevated plus maze, decreased open-field center activity following exposure to a predator cue, enhanced acoustic startle response and an enhanced conditioned fear. mTBI from a weight drop procedure has also been reported to decrease open-arm time in a plus maze without decreasing total distance (6 days post injury) and to enhance a conditioned fear (8 days post injury), while not affecting its extinction (Meyer et al., 2012). Thus, evidence exists that mTBI can alter the conditioned fear process.

We further evaluated the relationship between mTBI and conditioned fear in rats. We were particularly interested in determining whether mTBI could alter the process of extinction to a conditioned fear. An integral feature of many of the emotional conditioning processes involved in PTSD is that the conditioned stimuli are resistant to extinction. That is, stimuli that are associated with traumatic events continue to elicit intense emotional responses despite their repeated presentations in the absence of traumatic events (American Psychiatric Association, 2000). Thus, in addition to evaluating whether mTBI would alter the magnitude of a conditioned fear, we were also interested in evaluating whether mTBI would alter the extinction function within the conditioned fear paradigm.

To evaluate a conditioned fear, we used the method of Estes and Skinner (1941). In our implementation, previously neutral audio-visual stimuli are paired with aversive inescapable electric shock (IES) and, subsequently, elicit a conditioned emotional response which is generally described as fear. After conditioning, the audio-visual stimulus is embedded in an operant task and the resulting response suppression reflects the strength of the conditioned fear. Thus, the procedure allows for the concurrent evaluation of the conditioned fear and the general performance on the operant task. We evaluated extinction by repeated presentation of the audio-visual stimulus without IES over weekly test sessions for 2 months. It is notable that the conditioned suppression model is in contrast to conditioned freezing methods. While freezing would necessarily constitute response suppression, studies have shown that the suppression model involves additional conditioned fear processes (Amorapanth et al., 1999; Lee et al., 2005; McDannald, 2010; Pickens et al., 2010; McDannald and Galarce, 2011).

Clinically, the defining characteristics for the classification of mTBI are almost entirely based on signs or symptoms and include a relatively broad range of severity (Defense and Veterans Brain Injury Center, 2006). The translation to an infrahuman equivalent of mTBI is, therefore, notably challenging and is further complicated by the number and diversity of laboratory models in use (see review by DeWitt et al., 2013). In the present study, we used controlled exposure to BOP to produce mTBI. This model is reasonably well characterized (e.g., Long et al., 2009, 2010) and is particularly relevant as it closely represents a portion of the process resulting in a high prevalence of mTBI on the battlefield as documented from recent conflicts (Okie, 2005; Warden, 2006). In this regard, it is notable that BOP alone does not model the impact injuries that can accompany explosive blasts on the battlefield.

We chose a BOP of 74.5 kPa (10.9 psi) with the intention of producing an insult which could be considered to be in the low end of the mTBI range. The chosen pressure is less than those associated with gross pathology and specifically, neuronal pathology (e.g., Long et al., 2009; Readnower et al., 2010; Kamnaksh et al., 2011), but has been shown to produce behavioral effects such as anterograde amnesia (Ahlers et al., 2012). Additionally, we used three BOP exposures (1/day). This regimen has been used previously and was not found to produce neuronal pathology, but was found to produce behavioral effects including increased startle response and decreased maze movement (Elder et al., 2012). Finally, to exclude the traumatic stress that would be expected to accompany the BOP exposure, rats were anesthetized before both the BOP and sham exposures.

EXPERIMENTAL PROCEDURES

Animals

This study was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition. All procedures were reviewed and approved by the Institutes' Animal Care and Use Committees, and performed in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

Adult male Sprague–Dawley rats (Charles River, Wilmington, MA, USA) were used. Rats were individually housed in a temperature-controlled environment under a 12L:12D cycle (lights on at 06:00 h) and water was always available in the home cages. Body weights were maintained at approximately 325 g by food administered during experimental sessions and supplemental feedings (PMI Nutrition International, St. Louis, MO, USA) occurring several hours after experimental sessions. All rats were weighed daily, Monday–Friday.

Variable interval schedule of reinforcement

All rats were trained on a variable-interval 32 s schedule of reinforcement (VI). Sessions were conducted in twelve standard rodent operant conditioning chambers (model ENV-008 or equivalent, Med Associates, St. Albans, VT, USA) housed in ventilated, light- and sound-attenuating cubicles. Each chamber contained two response levers and a food trough attached to a food dispenser capable of delivering 45 mg food pellets (F0021, Bio-Serv, Frenchtown, NJ, USA). Each chamber also contained a house light mounted on the front wall near the ceiling, a stimulus light mounted

above each of the response levers and a Sonalert® tone generator (~2.8 kHz, model ENV-223 AM or equivalent, Med Associates, St. Albans, VT). Experimental events were controlled and monitored by a microcomputer, using Med-PC® control software (Med Associates, St. Albans, VT).

Although two levers were present in each chamber, only one lever produced food reinforcement. In this regard, an equal number of boxes were designated with the active lever on the left and on the right. Rats were initially trained to lever-press for food pellets under a continuous schedule of reinforcement where one lever press on the active lever always produced one food pellet. When lever pressing was maintained by food presentation, the contingencies were changed to the VI. The VI specifies that a single lever-press, following an average interval of 32 s, produces food reinforcement (i.e., a single food pellet). Interval values for the schedule were chosen pseudo-randomly, without replacement, from a set of values that followed a normal distribution (range = 0.8–127.9 s). Normal VI sessions were conducted without illuminating the house light or the stimulus lights above both levers. All sessions were 30 min in duration.

When responding under the VI was stable (as judged by inspection of the daily response rates), rats were assigned to a treatment group. In all cases, at least 60 training sessions were conducted before assignment. Assignment was balanced with respect to rate of responding with the objective that each treatment group would have similar average rates of responding.

Fear conditioning

Conditioning took place in a stainless steel chamber measuring 109 cm × 66 cm × 97 cm placed inside a ventilated, sound- and light-attenuating cabinet. The chamber contained a grid floor consisting of stainless steel rods running along the width and electric shock stimuli were presented through these rods. IES stimuli were generated by a Programmable Shocker (Lafayette Instrument Company, Lafayette, IN, USA, model HSMSCK). The device was used to output an isolated, scrambled (4-pole), constant current, electrical stimulus calibrated to 1.0 mA. The onset and duration of the shock stimulus was controlled by a laptop computer and associated interface equipment using custom software. The chamber also contained two house lights, four stimulus lights and a Sonalert ® tone generator, identical to those in the chambers used for the VI32 sessions.

Rats were placed in the chamber for a 35-min session in which 20 electric shock stimuli were presented at random times during the session with the exclusion of the first and last 2.5 min of the session and with at least 30 s between presentations. Each shock stimulus was 1.0 mA in intensity and 2.0 s in duration. A conditioned stimulus (CS) was presented 0.5 s prior to the onset of the shock stimulus and continued for the duration of the shock stimulus (i.e., 2.5 s total). The CS consisted of intermittently operating the Sonalert (0.35 s on, 0.15 s off), house light (0.25 s on, 0.25 s off) and stimulus lights (0.15 s on, 0.35 s off). For sham control conditions, sessions were conducted with all of the same stimuli and parameters except the shock stimuli were not presented.

Conditioned response (CR) testing

Sessions for the CR testing procedure were conducted in an identical fashion as the VI sessions described in Section 'Variable interval schedule of reinforcement' except that a CS (flashing lights and a pulsing tone) lasting 2.5 s was presented once during the session. The CS presented was essentially identical to that used during the fear conditioning procedure described in Section 'Fear conditioning'. That is, the CS consisted of intermittently operating the Sonalert (0.35 s on, 0.15 s off), house light (0.25 s on, 0.25 s off) and stimulus lights (0.15 s on, 0.35 s off) during the VI session. The CS was presented once during a session at a time randomly chosen from an array of times following a normal distribution but excluding the first 6 min and the last 8 min of the 30-min session.

BOP exposure

Rats were exposed to overpressure using a shock tube and air blast exposure under controlled conditions. The shock tube has a 12-inch circular diameter and is a 17.5-ft-long steel tube divided into a 2.5-ft compression chamber that is separated from a 15-ft expansion chamber. The compression and expansion chambers are separated by polyethylene Mylar sheets that control the peak pressure generated. The peak pressure at the end of the expansion chamber was determined by piezoresistive gauges specifically designed for pressure–time (impulse) measurements (Model 102M152, PCB, Piezotronics, Inc., Depew, NY, USA).

Rats were first anesthetized using an isoflurane gas anesthesia system consisting of a vaporizer, gas lines and valves, and an activated charcoal scavenging system. Rats were placed into a polycarbonate induction chamber, which was closed and immediately flushed with a 5% isoflurane mixture in air for 2 min. Rats were then placed into a cone-shaped plastic restraint device and then placed into the shock tube. Movement was further restricted during the blast exposure using restraint straps made from 1.5-cm-diameter flattened rubber tourniquet tubing. Three such straps were spaced evenly to secure the head region, the upper torso and lower torso while the animal was in the plastic restraint cone. The end of each strap was threaded through a toggle and run outside of the exposure cage where it was tied to prevent movement during the BOP exposure without restricting breathing. Rats were positioned with the head facing the blast exposure without body shielding to produce a full body exposure to the blast wave. Blast-exposed animals received 74.5-kilopascal (kPa) exposures equivalent to 10.9 pounds per square inch (psi). Using this system, the duration of the overpressure has been determined to be ~4.8 ms (Ahlers et al., 2012). Additionally, tests were performed to estimate variability in the maximum overpressure which was found to be 74.5 (±4.5) kPa, mean and SEM, respectively (Ahlers et al., 2012). One

exposure per day was administered for 3 consecutive days. For sham control conditions, rats were treated identically, including anesthesia and restraint, but did not receive a blast exposure.

Experimental series, groups and treatments

Extinction to a conditioned fear, with or without repeated BOP, was evaluated over the course of 8 weeks (see Fig. 1). Additionally, performance on the VI was evaluated during daily (Mon–Fri) sessions over the same time period. Four treatment groups were used (n = 10, each group): IES + Sham, IES + BOP, Sham + BOP, and Sham + Sham. All rats were first trained on the VI as described in Section 'Variable interval schedule of reinforcement'. Following training, rats were fear conditioned as described in Section 'Fear conditioning' by pairing IES with the CS (auditory and visual stimuli) for groups IES + Sham and IES + BOP. Sham control treatments for fear conditioning included the CS but without the IES (groups Sham + BOP and Sham + Sham). Fear conditioning took place during a single session following the VI session. On the 3 days following fear conditioning, rats in the treatment groups IES + BOP and Sham + BOP received exposure to BOP as described Section 'BOP exposure'. The exposures took place ~2 h before the VI session. For treatment groups IES + Sham and Sham + Sham, sham-BOP exposures were delivered. Four days after the last BOP or sham-BOP exposure (7 days after the CS + IES or CS + sham-IES) a single CS was presented during the VI session (i.e., CR testing) for all treatment groups. VI sessions were continued Mon-Fri, with a single CS presented during the VI session conducted on Mondays, for a total of eight CR tests. All rats were euthanized at the end of testing and tissue samples were taken as part of a larger proteomic study, to be presented separately.

Statistical analyses

When a response (i.e., lever press) occurred during the VI, the elapsed time within the session was recorded. From these data, the total number of responses and the rate of responding (responses per min) were calculated for each rat for the ''active'' lever (i.e., the lever producing food reward) and the inactive lever. Responding on the inactive lever was always very slow, typically accounting for less than one percent of the responses, and did not change systematically throughout the experiment. Therefore, these data were not analyzed further. Response rates on the active lever from the six sessions before CS + IES (or CS + sham-IES) were averaged and treated as a baseline control. Response rate data from subsequent sessions were converted to a percentage of the baseline values for each rat (i.e., percent of control).

Forty VI sessions were conducted following the CS + IES (or CS + sham-IES) presentation and response rate measures from these sessions were averaged into eight blocks of five consecutive sessions for analysis. Additionally, individual sessions were analyzed after the CS + IES (or CS + sham-IES) presentation and up to the first CR test session. This period of performance included VI sessions conducted after each of the BOP (or sham-BOP) exposures.

To evaluate the strength of the CR for fear conditioning, suppression indices were calculated according to the formula: (response rate before the CS − response rate after the CS)/(response rate before the CS + response rate after the CS). This measure yields a value of 0 when there is no response suppression due to CS presentation and a value of 1 when responding is completely suppressed by CS presentation. We calculated suppression indices for both ±1 and ±3 min around the CS. While we expected these measures to be correlated, their use maximized the quantification of the strength of the conditioned fear and also reduced the possibility of a restriction due to a ceiling effect that might occur if responding was completely suppressed during a short interval following the CS. Suppression indices were calculated for each rat for each CR test session.

Inferential statistics were calculated using the SAS (Cary, NC) statistical software package. A two factor (treatment by time) mixed model analysis of variance (ANOVA) (with a Satterthwaite approximation for the denominator degrees of freedom) was performed for the VI response rate and suppression index measures. The procedure allows for the specification of the covariance structure. Based upon measures of fit (e.g.,

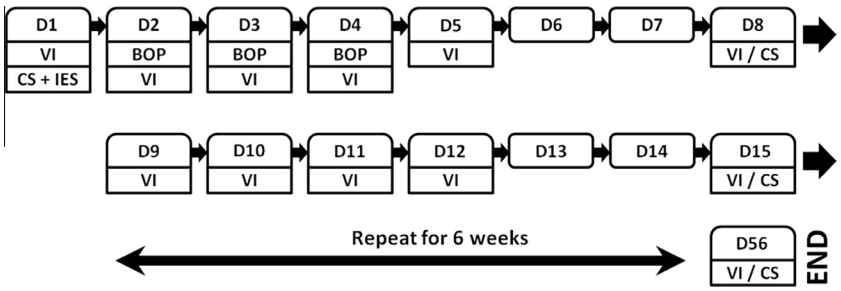

Fig. 1. Study design. All rats were first trained on the variable interval schedule of reinforcement (VI) and VI sessions continued to be conducted daily (Mon–Fri) throughout the study. Fear conditioning was implemented by pairing an auditory and visual conditioned stimulus (CS) with inescapable electric shock (IES). Following conditioning, exposures to blast overpressure (BOP) were presented on 3 consecutive days. Tests to evaluate the conditioned response (CR) from fear conditioning were performed by presenting the CS once during the VI session and measuring the resulting degree of response suppression. CR test sessions were conducted every 7 days after fear conditioning during the course of 8 weeks.

AIC, AICC and BIC), a compound symmetry model was used for the VI response rate data and an autoregressive model (AR1) was used for the suppression index data. Following ANOVA, selected contrasts were performed. In all cases, the criterion for statistical significance was set at $p < .05$.

RESULTS

Responding under the VI schedule was acquired by all rats. Baseline measures of responding on the active lever (i.e., the lever producing food reinforcement), defined as the average of the last six sessions conducted before exposure, for the treatment groups ($n = 10$ each group) were as follows (mean ± SEM responses per min): IES + Sham = 51.4 ± 6.9, IES + BOP = 56.5 ± 10.3, Sham + BOP = 50.1 ± 3.7, Sham + Sham = 50.7 ± 5.1. Fig. 2 shows performance on the VI from the last baseline session through the first CR test. ANOVA evaluating VI performance during the five sessions after fear conditioning and including the session with the first CR test revealed no significant effects for group ($F[3,36] = 0.96$, $p > .05$) or the group by session interaction ($F[12,144] = 0.75$, $p > .05$) but did reveal a significant main effect for session ($F[4,144] = 8.71$, $p < .001$). Tests of effect slices for the session factor showed significant effects for the IES + BOP ($F[4,144] = 3.32$, $p < .01$) and Sham + Sham ($F[4,144] = 3.96$, $p < .01$) treatment groups but not for the IES + Sham ($F[4,144] = 1.38$, $p > .05$) or Sham + BOP ($F[4,144] = 2.31$, $p > .05$) groups. Although there was not a significant main effect for groups, we were particularly interested in whether any changes could be attributed to a common treatment of IES or BOP presentation. Thus, we evaluated, but found

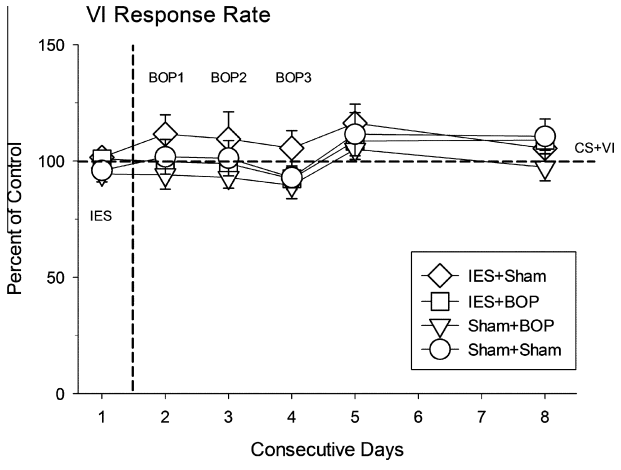

Fig. 2. Performance on the VI schedule of reinforcement during six consecutive test sessions. CS + IES (or sham) occurred following the test session on day 1. BOP (or sham) occurred ~2 h before the test sessions on days 2–4. The CS alone was presented during the session on day 8. Ordinate: response rate as a percentage of control (determined as the average response rate from six baseline sessions). Abscissa: consecutive days. Each point represents the mean (±SEM) from 10 rats. Dashed horizontal line indicates control rate of responding. Points to the left of the vertical dashed line represent the last baseline session.

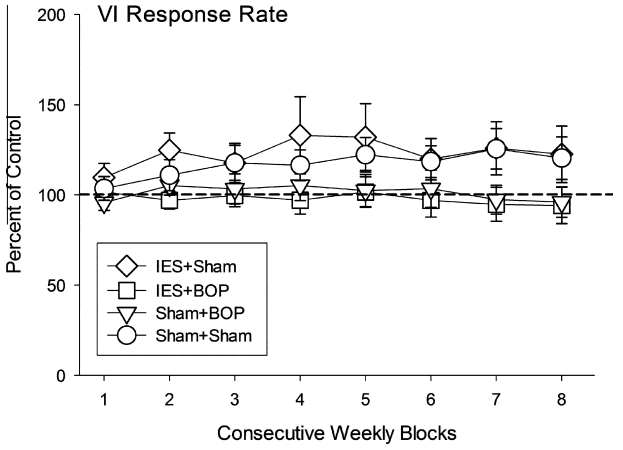

Fig. 3. Performance on the VI schedule during 8 consecutive weeks. Ordinate: response rate as a percentage of control (determined as the average response rate from six consecutive baseline sessions). Abscissa: consecutive blocks. Each point represents the mean (±SEM) from 10 rats and each block contains the average response rate from five sessions. Dashed horizontal line indicates control rate of responding.

no significant effects for, contrasts comparing groups receiving BOP (IES + BOP and Sham + BOP) vs. no BOP (IES + Sham and Sham + Sham) ($F[1,36] = 1.83$, $p > .05$), and IES (IES + BOP and IES + Sham) vs. no IES (Sham + BOP and Sham + Sham) ($F[1,36] = 1.06$, $p > .05$).

Fig. 3 shows VI performance over 8 weeks beginning with the session following CS + IES (or CS + sham-IES). In general, performance on the VI was maintained near baseline levels in all groups, although some deviations from baseline were present. ANOVA showed no main effects for groups ($F[3,36] = 1.77$, $p > .05$) and no groups by session interaction ($F[21,252] = 1.21$, $p > .05$), but did show a significant main effect for sessions ($F[7,252] = 2.07$, $p < .05$). Analysis of effect slices for sessions revealed a significant effect only for the IES + Sham group ($F[7,252] = 2.44$, $p < .05$). No significant effect was found for contrasts that compared IES groups vs. no IES groups (IES + Sham and IES + BOP vs. Sham + BOP and Sham + Sham, $F[1,18] = .03$, $p > .05$) or BOP groups vs. no BOP groups (IES + BOP and Sham + BOP vs. IES + Sham and Sham + Sham, $F[1,18] = 5.03$, $p > .05$).

No grossly observable effects from the IES or from BOP exposures were noted. All rats appeared normal shortly following anesthesia and BOP and shortly following IES. Furthermore, all rats appeared normal during weighing and handling throughout the experiment. Fig. 4 presents the extinction functions for conditioned fear, as evidenced by the degree of response suppression (suppression index for ±1 min [top] and ±3 min [bottom]), for the four treatment groups during the eight consecutive weekly CR tests. As expected, presentation of the CS during the VI session initially produced substantial response suppression in treatment groups where the CS had been previously paired with IES (i.e., IES + Sham and IES + BOP). Also as expected, the CS produced very little response

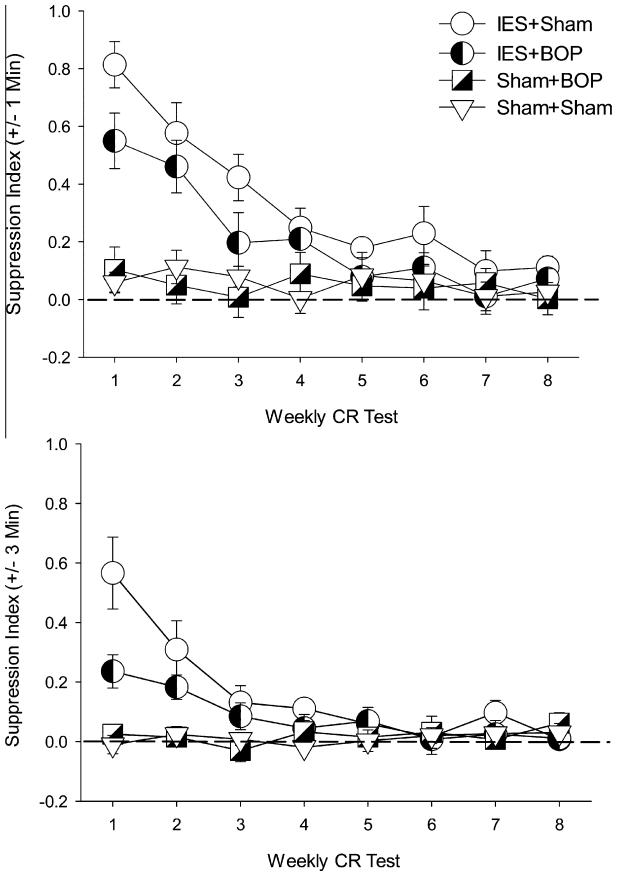

Fig. 4. Extinction of conditioned suppression. Ordinates: suppression indices (±1 min, top and ±3 min, bottom) during eight weekly CR test sessions. Abscissas: consecutive weeks. Each point represents the means (±SEM) from 10 rats. Dashed horizontal lines represent a suppression index value of 0 indicating the same rate of responding before the CS as after the CS (i.e., no response suppression).

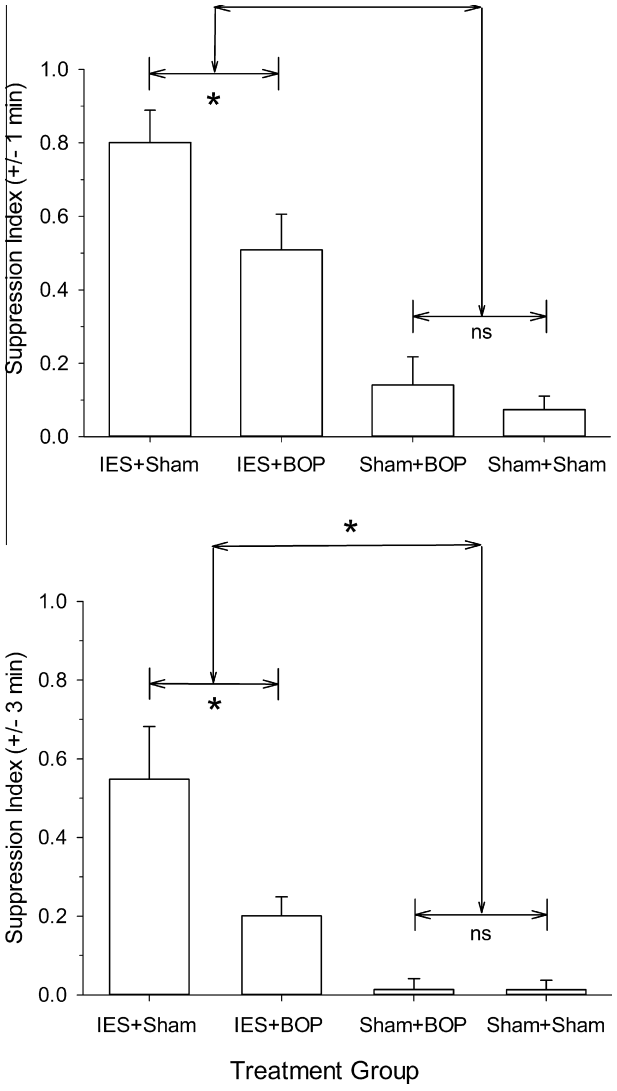

Fig. 5. Conditioned suppression during the first CR test administered 7 days after CS + IES pairing and four days after the last BOP exposure. Ordinates: suppression indices (±1 min, top and ±3 min, bottom). Abscissas: four treatment groups: IES + Sham, IES + BOP, Sham + BOP and Sham + Sham. Bars represents the mean (+SEM) from 10 rats. Asterisks indicate a statistically significant difference (contrasts following ANOVA, ps < .05) and "ns" indicates comparison not statistically significant.

suppression in treatment groups where the CS had not been paired with IES. In general, when present, the response suppression produced by the CS was greater for the ±1 min index as compared with the ±3 min index. For the ±1 min suppression index (Fig. 4, top panel), ANOVA showed a significant main effect for treatment group ($F[3, 82.6] = 22.62$, $p < .001$), CR session ($F[7, 180] = 10.22$, $p < .001$) and the treatment group by CR session interaction ($F[21, 180] = 2.87$, $p < .001$). Similarly, for the ±3 min suppression index (Fig. 4, bottom panel), ANOVA showed a significant main effect for treatment group ($F[3, 61.3] = 9.95$, $p < .001$), CR session ($F[7, 180] = 6.82$, $p < .001$) and the treatment group by CR session interaction ($F[21, 180] = 3.78$, $p < .001$). For both the IES + Sham and IES + BOP treatment groups, the conditioned fear diminished with continued presentation of the CS as can be seen by a reduction in both suppression indices during the later CR test sessions. Analyses of the effect slices for CR sessions for the ±1 min suppression index showed significant effects for both the IES + Sham ($F[7, 180] = 11.39$, $p < .001$) and IES + BOP ($F[7, 180] = 6.80$, $p < .001$), but not for the Sham + BOP ($F[7, 180] = .31$, $p > .05$) and Sham + Sham ($F[7, 180] = .034$, $p > .05$). The same profile of significance was found for the ±3 min suppression index (IES + Sham, $F[7, 180] = 14.82$, $p < .001$; IES + BOP, $F[7, 180] = 2.66$, $p < .02$; Sham + BOP, $F[7, 180] = .49$, $p > .05$; Sham + Sham, $F[7, 180] = .17$, $p > .05$). As can be seen from Fig. 4, conditioned fear in the IES + Sham group was, on average, greater than that in the IES + BOP group. Contrasts between these two groups across CR sessions showed a significant difference for both the ±1 min index ($F[1, 82.6] = 9.24$, $p < .005$) and the ±3 min index ($F[1, 61.3] = 6.42$, $p < .02$). The difference between these two groups is also illustrated in Fig. 5 which shows the degree of suppression (suppression index for ±1 min [top] and ±3 min

[bottom]) for the four treatment groups during the first CR test. Contrasts comparing the treatment groups at this time point show a significant difference in suppression between the IES + Sham-S and IES + BOP treatments for both the ±1 min (top panel, $F[1,268] = 7.54$, $p < .01$) and the ±3 min (bottom panel, $F[1,223] = 26.54$, $p < .001$) indices. Additionally, both the IES + Sham and IES + BOP groups were significantly different than both the Sham + BOP and Sham + Sham groups for the ±1 min ($Fs[1,223] \geqslant 21.48$, $ps < .001$) and the ±3 min ($Fs \geqslant 10.75$, $ps < .01$) indices. The Sham + BOP and Sham + Sham groups did not differ significantly for either index (±1 min index, $F[1,268] = .22$, $p > .05$; ±3 min index, $F[1,223] = .30$, $p > .05$).

DISCUSSION

We trained rats on an operant schedule of food reinforcement and then trained a conditioned fear by pairing IES with an audio-visual stimulus. Subsequently, rats were exposed to repeated mTBI from BOP. Presentation of the CS after pairing with IES took place in a different context than the IES and produced a robust conditioned fear as quantified by both the ±1 min and ±3 min suppression indices. BOP reduced the degree of conditioned suppression. That is, as compared to sham-BOP controls, BOP decreased the expression of a conditioned fear that was trained prior to exposure. There are several possible interpretations of this result. First, it could be argued that the BOP produced sensory damage to the auditory and/or visual system such that the perception of the CS was altered in exposed rats. While BOP, using a similar procedure as in the present study, has been reported to produce visual system degeneration, it did so only at substantially higher pressures (104–173 kPa) and a pressure of 84 kPa, which is greater than that used in the present study, did not result in any visual system pathology (Petras et al., 1997). Additionally, the exact regimen of BOP used in the present study was not found to produce any changes in the prepulse inhibition of a startle response, suggesting that auditory perception was also not impaired (Elder et al., 2012). Therefore, it is not likely that sensory damage due to BOP was responsible for the observed difference between the IES + Sham and IES + BOP treatment groups.

A second interpretation of this result is that the BOP produced a retrograde amnesia. In this regard, it is notable that the BOP exposures took place beginning at ~22 h after the IES. It is likely that enough time had elapsed for memory consolidation of the event to have occurred (McGaugh, 2000). Thus, the amnesic effect would not have been through the disruption of memory consolidation processes such as when the insult takes place shortly after the conditioning event. Furthermore, a single BOP at the same and at a greater pressure than used in the present study did not produce an amnesic effect when exposure immediately followed a passive avoidance task (Ahlers et al., 2012). Typically, more severe injuries are required to produce a retrograde amnesia for events already presumed to be consolidated into long-term memory (e.g., Chen et al., 2009). It is also notable that BOP-exposed animals did show a conditioned fear, although to a lesser degree than the sham-BOP treatment group. Thus, the retrograde amnesia would have to be characterized as partial.

While a retrograde amnesia cannot be ruled out, we propose that the BOP exposure more likely decreased behavioral inhibition. That is, responding on the VI task is maintained by food reinforcement and the schedule of reinforcement exerts a degree of stimulus control (i.e., represents a motivated task). Following pairing with the IES, the CS elicits a CR (i.e., conditioned fear) which is in conflict with responding on the VI task. In this sense, the CS serves as an inhibitory or "stop" signal. The BOP exposure appears to have decreased the inhibitory control exerted by the CS although responding on the VI was unaffected. While further studies are needed to confirm this possibility, it is notable that failures of inhibitory control behaviors are integral features of many psychiatric disorders and the mechanism of an inhibitory control system in rats has been the subject of substantial study (see review by Eagle and Baunez, 2010). Furthermore, deficits in behavioral inhibition have been observed in patients following TBI (Dimoska-Di Marco et al., 2011; Dockree et al., 2006; O'Keeffe et al., 2007).

The decreased expression of a conditioned fear produced by BOP in the present study represents a functional deficit. That is, the optimal conditioned fear response is best represented by the IES + sham treatment and a substantial deviation from that response can reasonably be interpreted as an adverse outcome. Our results, however, are in stark contrast to previous results showing that mTBI produced an exaggerated conditioned fear as compared to controls (Elder et al., 2012; Meyer et al., 2012; Reger et al., 2012). A major difference between the former studies and the present study is that the conditioned fear in the present study was established (and presumably consolidated into long-term memory) before the mTBI exposures. In the former studies, conditioning took place days or weeks after the mTBI insults. A second difference is that we used a conditioned suppression procedure to evaluate conditioned fear whereas the former studies used a conditioned freezing procedure. Freezing, by definition, prevents lever pressing and previous studies have shown that conditioned freezing and conditioned suppression are correlated (e.g., Pickens et al., 2010). Additional studies using lesioning have shown that the dependencies of these two behaviors on the basolateral and central nuclei of the amygdala, and the ventral periaqueductal gray differs (Amorapanth et al., 1999; Lee et al., 2005; McDannald, 2010;; McDannald and Galarce, 2011). Therefore, the different results in the present study may reflect qualitatively different effects of mTBI on conditioned fear.

We were particularly interested in whether BOP would alter the course of extinction, independently of whether it affected the initial expression of conditioned fear. In this

regard, both IES + Sham and IES + BOP rats showed orderly extinction functions (Fig. 4) that appeared to be essentially parallel. Repeated exposure to the CS produced progressively less suppression in both groups. There was a statistically significant difference in the extinction functions between the two groups, but that difference is consistent with the decreased initial conditioned fear response produced by BOP. Furthermore, both groups reached near zero values for the suppression indices that were equivalent to groups that had not received CS + IES pairing. We, therefore, conclude that BOP did not delay or facilitate extinction to a conditioned fear, although it did alter the magnitude of its expression. This result is consistent with a previous study (Meyer et al., 2012) that showed that mTBI from weight drop did not alter extinction of a conditioned fear, while altering (in this case increasing) the initial expression of the conditioned fear. The finding is relevant to understanding the relationship between mTBI and PTSD since the persistence of an emotional response to previously neutral stimuli that have been associated with trauma is an integral feature of some PTSD symptoms (American Psychiatric Association, 2000). Additionally, individuals with PTSD have shown resistance to extinction in fear conditioning in the laboratory (e.g., Blechert et al., 2007; Wessa and Flor, 2007). In this regard, our results do not support a predisposing or causative relationship between mTBI and PTSD.

Neither the BOP nor IES manipulations had a robust or systematic effect on responding on the VI. In general, responding was maintained during sessions occurring only a few hours after the BOP sessions. Previous studies have shown that operant behavior is a reasonably sensitive measure of performance and we have previously used such procedures to evaluate the effects of drugs, toxins and ischemic injury (Genovese et al., 1988, 1992, 1993, 2006). Moreover, TBI, from a fluid percussion injury, has been shown to decrease responding on a food-maintained operant task (Gorman et al., 1993). The mTBI exposure in the present study was clearly insufficient to disrupt performance on the task taking place several hours later and clearly suggests that the intensity of the repeated BOP used was, in fact, a mild insult. We also did not observe the BOP to produce any long-term or delayed disruption on the VI task as we evaluated performance for approximately 2 months following the BOP. In this respect, we did not observe any delayed neurobehavioral effects known to occur in patients following TBI (e.g., Gualtieri and Cox, 1991), further suggesting that the BOP used in the present study is a mild insult.

As expected, rats that did not receive IES did not show any conditioned fear. The occurrence of the CS during the VI session, however, did produce a small degree of disruption in groups not receiving IES (Sham + BOP and Sham + Sham) as shown by suppression indices that were consistently above zero. CS presentation, however, constituted an abrupt and unpredictable disruption of the normally dark and quiet VI environment. In this regard, the response to the CS in rats not previously receiving CS + IES pairings can be considered a startle response. There was no difference in this startle response, however, between groups receiving BOP or sham-BOP. It is also notable that the anesthesia used in the present study did not appear to affect any of the performance measures. Isoflurane was used to eliminate any traumatic stress from the BOP and also to reduce any movement that might produce variability in the effects of the BOP, but the same anesthetic regimen was used in all treatment conditions.

The present study demonstrates that, in rats, a behavioral deficit in the expression of conditioned fear can be caused by mTBI from BOP. The BOP intensity used to produce mTBI was below that shown previously to produce gross pathology and specifically, neuronal pathology and can be considered a mild insult. While the BOP produced a deficit in the expression of a conditioned fear, the deficit was observed as a reduction in the impact of the conditioned fear as compared to IES + sham controls. Taking into account the difference in initial impact, extinction to the conditioned fear appeared to occur normally in both IES + BOP and IES + Sham groups. It is notable that these results, demonstrating that the mTBI produced a reduction in the expression of a conditioned fear while not increasing resistance to extinction, are not in the direction of an effect that is analogous to PTSD. While affecting the conditioned fear, the BOP did not produce acute deficits on general performance as measured by the VI, even when evaluated hours after exposure. Furthermore, no delayed effects on the VI were caused by the mTBI as no systematic changes in long-term performance on the VI were observed over 2 months of post-mTBI evaluations. In this regard, the results suggest that the intensity of the mTBI was, indeed, mild and is likely at the lowest end of the mTBI continuum. Results from this study are, of course, limited to a single conditioned fear procedure and further research is required to extend the findings to additional behavioral process that are integral to animal models of PTSD. Taken together, however, these results augment previous studies evaluating the behavioral effects of mTBI and particularly the possible relationship between mTBI and PTSD.

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